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FEELING THE FORCE: ROLE OF AMOTL2 IN NORMAL DEVELOPMENT AND CANCER

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Karolinska Institutet

Stockholm 2019

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Published by Karolinska Institute.
Printed by: US-AB Stockholm

ISBN 978-91-7831-426-3

Feeling the force: Role of AmotL2 in normal development and cancer.

THESIS FOR DOCTORAL DEGREE (Ph.D.)

J3:04, Torsten N Wiesel, U410033310, Bioclinicum, Karolinska University Hospital, Solna, Stockholm

Friday, April 12th, 2019 at 13:00

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To my mom and dad

"Real knowledge is to know the extent of one's ignorance."

(*Confucius*)

ABSTRACT

Cells that fabricate the body, dwell in a very heterogeneous environment. Self-organization of individual cells into complex tissues and organs at the time of growth and revival is brought about by the combinatory action of biomechanical and biochemical signaling processes. Tissue generation and functional organogenesis, requires distinct cell types to unite together and associate with their corresponding microenvironment in a spatio-temporal manner. Furthermore, the cell-cell and cell-extracellular matrix (ECM) contact points are tethered together by the cytoskeletal protein network within the cell, which in turn connects to the nucleoskeleton. These uninterrupted networks from cell junctions to nucleus enable the cells to sense it's surrounding. Recently it was found that relay of mechanical cues from cell exterior to interior (Mechanotransduction) is key to cellular fate determination and also behavior of an individual cell. However, deregulation of this signal processing has been reported to cause adverse consequences, for example increased stiffness in the ECM can result in loss of tissue architecture and promotes tumor progression. Mechanotransduction of the cell and its ECM have been extensively studied and elucidated to be important in driving various pathophysiological processes. However, the mechanisms underlying force transmission *via* cell-cell junctions and their role in morphogenesis and maintaining homeostasis still remain elusive.

In this thesis, we have reported Angiomotin-Like-2 (p100AmotL2) as a novel linker protein, connecting VE/E-cadherin at the adherens junction (AJ) and the nucleus. Furthermore, we show that p100AmotL2 enables radial actin filament organization, which is essential for force generation/transmission required for various developmental processes such as aortic lumen expansion and blastocyst hatching. In paper 3 of this thesis, we identified the stress responsive shorter isoform of AmotL2 (p60AmotL2), which acts in a dominant negative fashion, by disrupting the VE/E-cadherin/p100AmotL2/nucleus mechanotransduction. Additionally, we also show that p60AmotL2 weakens cell-cell cohesion and alters nuclear integrity contributing to loss of tissue architecture and promote cellular invasion. The normal physiological function of p60AmotL2 has not yet been revealed, in Paper 4 of this thesis; we have elucidated novel molecular mechanism of p60AmotL2 in maintaining tissue homeostasis by promoting apical extrusion of cells. As cancer is known to hijack several physiological pathways, it is tempting to speculate that cancer cells might hijack p60AmotL2 mediated extrusion process to invade the surrounding tissues.

LIST OF SCIENTIFIC PAPERS

I. **AmotL2 integrates polarity and junctional cues to modulate cell shape.**

Sara Hultin*, **Aravindh Subramani***, Sebastian Hildebrand, Yujuan Zheng, Arindam Majumdar and Lars Holmgren. Sci Rep. 2017; 7: 7548.

II. **The E-cadherin/AmotL2 complex organizes actin filaments required for epithelial hexagonal packing and blastocyst hatching.**

Sebastian Hildebrand, Sara Hultin, **Aravindh Subramani**, Sophie Petropoulos, Yuanyuan Zhang, Xiaofang Cao, John Mpindi, Olli Kalloniemi, Staffan Johansson, Arindam Majumdar, Fredrik Lanner & Lars Holmgren. Sci Rep. 2017; 7: 9540

III. **Uncoupling of the Nuclear LINC-complex from E-cadherin/radial actin filaments trigger single cell migration and invasion.**

Aravindh Subramani, Tomas Friman, Yuanyuan Zhang, Weyingqi Cui, Malgorzatta Lekka, VaniNarayanan, Daniel Conway and Lars Holmgren.

IV. **p60AmotL2 induces epithelial apical extrusion.**

Aravindh Subramani, Weiyingqi Cui and Lars Holmgren.

*These authors contributed equally

LIST OF ABBREVIATIONS

Amot	Angiomotin
AmotL1	Angiomotin Like 1
AmotL2	Angiomotin Like 2
AMP	Adenosine Monophosphate
ATP	Adenosine Triphosphate
ADP	Adenosine Diphosphate
ABP	Actin binding protein
AFM	Atomic Force Microscopy
aPKC	Atypical Protein Kinase C
CAR	Coxsackievirus and Adenovirus Receptor
Crb3	Crumbs homolog 3
Caco2	Human colonic adenocarcinoma cells
cDNA	Complementary DNA
DNA	Deoxyribonucleic Acid
Dlg	Discs large
DCIS	Ductal Carcinoma In-situ
dsRed	Discosoma sp. Red fluorescent protein
E-cadherin	Epithelial cadherin
ECM	Extracellular Matrix
EGFP	Enhanced Green fluorescent protein
EMT	Epithelial to Mesenchymal Transition
ERK	Extracellular signal-regulated kinase
EGF	Epidermal growth factor
FA	Focal Adhesion
FGF	Fibroblast growth factor
FAK	Focal Adhesion Kinase
FRET	Forster Resonance Energy Transfer
F-actin	Filamentous actin
GFP	Green fluorescent protein
GAP	GTPase Activating Protein

gata1	GATA binding protein
G-actin	Globular actin
GTP	Guanosine Triphosphate
hpf	Hours post fertilization
HGF	Hepatocyte Growth Factor
IGF	Insulin-like-Growth Factor
ICM	Inner cell mass
INM	Inner Nuclear Membrane
JAM	Junctional Adhesion Molecule
Kdrl	Kinase insert domain receptor like
KASH	Klarsicht, ANC-1, Syne Homology
LATS	Large Tumor Suppressor kinase
Lgl	Lethal (2) giant larvae
LINC	Linker of Nucleoskeleton and Cytoskeleton
MDCK	Madine Darby canine kidney
MAGI	Membrane Associated Guanylate kinase protein
MAPK	Mitogen Activated Protein Kinase
MET	Mesenchymal to Epithelial Transition
MO	Morpholino
mRNA	Messenger RNA
MST	Mammalian Ste20-like Serine/Threonine kinase
Mupp1	Multiple PDZ domain protein 1
Ms1	Mile Sven 1
MLP	Myosin Light chain
NE	Nuclear Envelope
ONM	Outer Nuclear Membrane
Pals	Proteins associated with caenorhabditis elegans Lin7 protein
Par	Partition defective
Patj	Pals1 associated tight junction protein
PDZ-domain	PSD95, Dlg1 and ZO-1 binding domain
RFP	Red Fluorescent Protein

Rac	Ras-related C3 botulinium toxin substrate
Ras	Rat sarcoma viral oncogene homolog
Rho	Ras homolog family member
ROCK	Rho associated Coiled-coil containing protein kinase
Scribble	Scribbled planar cell polarity protein
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SUN domain	Sad1p, UNC-84 domain
TJ	Tight junction
TE	Trophoectoderm
TGF	Transforming Growth Factor
TGF-β	Transforming Growth Factor beta
TAZ	Transcriptional co-Activator with PDZ-binding motif
TRAPP	Trafficking Protein Particle
Twist	Twist related protein
VE-cadherin	Vascular Endothelial cadherin
YAP	Yes Associated protein
ZO-1	Zonula Adherens 1

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Background

The building blocks of the human body

Cells form the structural and functional unit of all organisms, however, in multicellular organisms, single cells cannot be able to perform all the vital physiological functions. Hence the cells unite to form tissues, whereas the tissues unite together to form organs and in turn organs unite to form an organ system, which is the basis to form living creatures. The organ systems in the human body are composed of various cell types such as epithelial, endothelial, neuronal and muscle cells, which interact in a highly synchronized manner among the same cell type and other cell types to carry out a number of complex functions. Epithelia are a specialized type of cells performing important physiological functions such as covering exterior surfaces of the body, lining the cavities as in the gastro-intestinal tract, lungs, kidney etc and thus creating a physical barrier between organs and the external milieu. Besides forming a protective covering, epithelial cells also perform other functions such as absorption of nutrients from the digestive system, secretion of hormones, production of digestive enzymes, excretion in the form of sweat and the exchange of gases in lungs (Guillot and Lecuit, 2013). The epithelial cells are classified into simple and stratified epithelia, where the simple epithelium is made of a single layer and the latter contains two or more layers. These two types of epithelia are further classified into squamous, cuboidal and columnar based on their morphology (Gibson and Gibson, 2009; Hagios et al., 1998). Understanding the mechanisms underlying epithelial organization is of highest importance as the majority of human cancers are epithelial derived and it also accounts for 80% of the overall cancer death around the world.

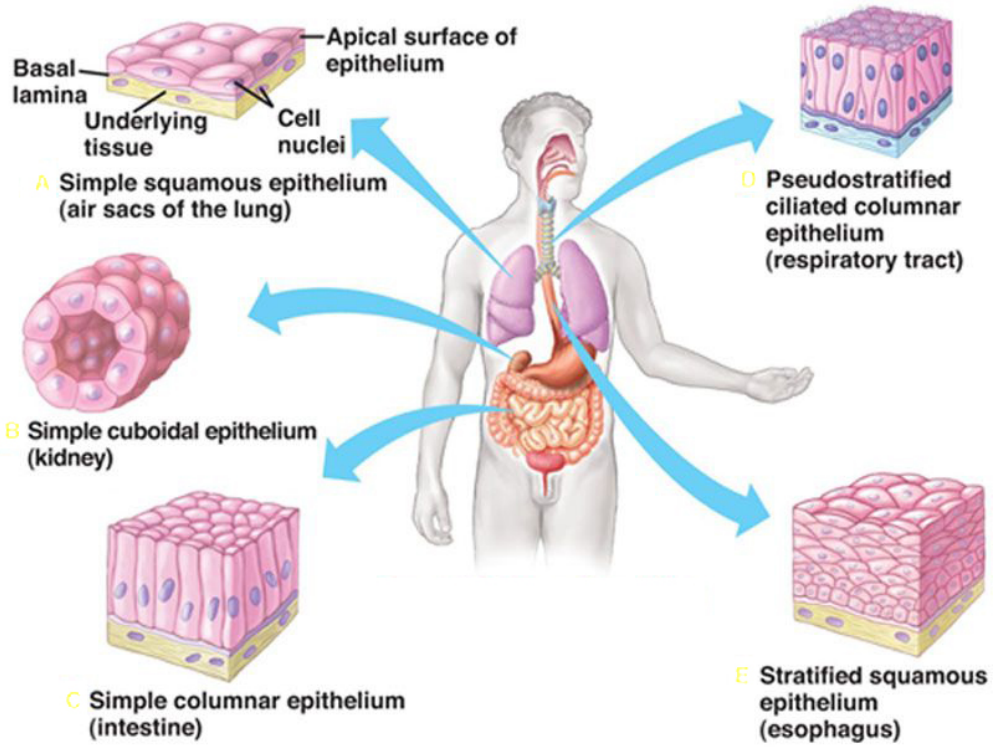

Figure 1:Different types of epithelial cells lining various organs. Epithelial cells are of various shapes and are classified as squamous, cuboidal and columnar epithelium based on their shape. The epithelial cells line various organs such as lungs, kidney, intestine, the respiratory tract, esophagus etc. Adapted from https://www.smartsciencepro.com/animal-tissues/

Mechanisms of Epithelial sheet formation

Epithelial cells form an organ in organized layers or sheets of cells. Prominent examples of this can be seen in the skin or cysts and tubules found within internal organs. In order to be able to form these structures, cells undergo various biological processes. Such an organization is complex and highly coordinated, involving growth factor signaling, cell-cell contact, cell-extra cellular matrix (ECM) interactions and polarization of cells. The cell-cell contacts, cell-ECM interactions, polarity and cytoskeletal organization are considered to be the key events in the epithelial organ formation (Gibson and Gibson, 2009; O'Brien et al., 2002). In the following paragraphs, the different types of cell adhesomes, cytoskeletal subtypes and fundamental mechanisms for establishing cell polarity will be discussed in detail.

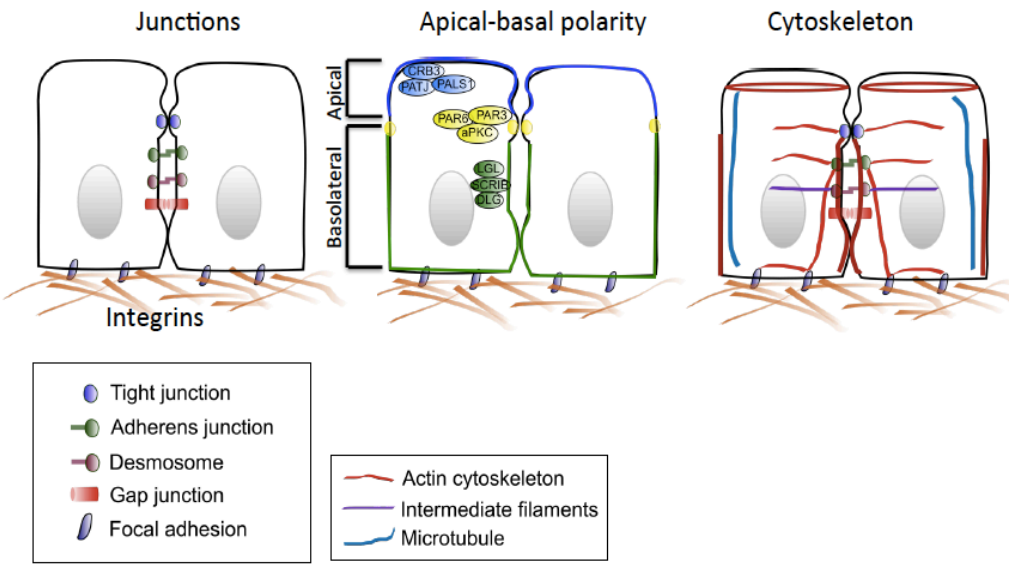

Figure 2: Cellular events essential for epithelial sheet formation: Cell-cell and cell-ECM contacts, apical-basal polarity and actin cytoskeletal organization are required to form a functional epithelial sheet.

Protein complexes required for the formation of functional tissue architecture

Cell-cell adhesome

Cells are connected via interaction sites found at cell-cell junctions and these contacts are considered to initiate epithelial organization. Cell-cell junctions are classified based on the molecules involved in forming the junctions and includes the adherens junctions (AJ), tight junctions (TJ), desmosomes, hemi desmosomes and gap junctions (Cooper, 2000a; Miyake et al., 2006). AJ`s are crucial for maintaining cell-cell adhesions. Adhesion proteins and their receptors facilitate formation of these junctions and subsequent interactions. The AJ is the first connection to be formed between cells. One of the well-studied adhesion proteins to establish a stable epithelium is E-cadherin. E-cadherin is a calcium dependent adhesion molecule connecting the epithelial cells through homophilic interactions situated at the AJ`s. The extracellular domain of E-cadherin is responsible for connecting cell-cell contacts to the actin cytoskeleton within the cell (Baum and Georgiou, 2011; Desai et al., 2013; Grieve and Rabouille, 2014; Gumbiner, 2000). Interactions between cells facilitate the formation of TJ´s to maintain tight barriers by restricting the free passage of water and small

molecules. The TJ´s are composed of protein complexes like occludins, claudins, tricellulin, junctional adhesion molecules (JAMs), coxsackievirus and adenovirus receptor (CAR), CXADR like membrane protein (CLMP) and crumbs homolog 3 (CRB3) (Aijaz et al., 2006; Balda and Matter, 2008; Miyake et al., 2006).

The desmosomes are comprised of specialized cadherins such as desmogleins and desmocollins and are therefore more robust, but less dynamic when compared to the AJ and TJ`s because of their association with the intermediate filaments rather than the actin cytoskeleton (Vasioukhin et al., 2000). Desmosomes are described as 'button-like' structures that weld the cells together and are abundantly found in epithelial cells and the myocardium. The different types of desmosomes are keratins in epithelial cell and desmin in the myocardium (Alberts B, 2002). The Gap junctions are quite different from the other junctional type as they are formed by the clustering of connexin based intercellular channels (Wei and Huang, 2013). Gap junctions are important for the exchange of small metabolites like glucose molecules, ion channels and secondary messengers like cAMP and cGMP (Mese et al., 2007; Miyake et al., 2006).

Cell-Matrix adhesome

The ECM is a complex array of matrix proteins and non-matrix molecules including collagens, glycoproteins, proteoglycans, laminins, fibronectin, proteolytic enzymes and growth factors which are cross-linked into insoluble complexes (Hynes and Naba, 2012). Epithelial cells connection to the underlying ECM is mediated by integrin binding to their ligands provided within the ECM. The integrins are a well-studied transmembrane heterodimer protein family, which form clusters at their intracellular domains upon activation (Changede and Sheetz, 2017). These integrin clusters are important for interaction with the actin cytoskeleton through various actin-binding proteins (ABP) such as Vinculin, Talin, Filamin, Tensin and α-Actinin. The point at which the integrin cluster and the actin cytoskeleton connect/interact is termed a focal adhesion (FA). Interactions between the cell and ECM are important in actin re-organization, establishing cell polarity and are also involved in the regulation of

several physiological functions including cell growth, differentiation and migration (Brakebusch and Fassler, 2003; Burridge and Chrzanowska-Wodnicka, 1996). Several other interaction points between cell-ECM have been identified in the past decade such as focal complexes, fibrillar adhesions and three dimensional matrix adhesions (Cukierman et al., 2001; Nobes and Hall, 1995; Zamir et al., 2000). These complexes exhibit similarity with the FA complexes with small variations in their structure and constituent proteins (Chen et al., 2004).

Apical-basal polarity protein complexes

Cell polarization is an evolutionarily conserved process, which is essential for epithelial organization and organ formation. Cell polarity can be defined as the asymmetrical distribution of different cellular components including lipids, carbohydrates, proteins, organelles and the cytoskeleton within the cellular compartment (Bryant and Mostov, 2008; Drubin and Nelson, 1996). Epithelial cells are polarized in three different fashions; front-rear, apico-basal or planar. Front-rear polarity is mobilized during directed cell migration during various physiological processes such as tissue repair (Zallen, 2007). Planar polarity refers to the cumulative arrangement of cell polarity over the tissue plane (eg follicles and the scales of a fish) and is essential during the elongation of tissues during morphogenesis (Devenport, 2014; Zallen, 2007). Apico-basal polarity is pertinent in the formation of cysts and lumenisation. The establishment of the cell-cell and cell-ECM contact mediates the formation of apical and baso-lateral domains in the cell, which are then followed by the distribution of different protein complexes to their respective localized domains (Nelson, 2003; O'Brien et al., 2002; Yeaman et al., 1999). Important protein complexes involved in the establishment of polarity include Par, Crumbs and Scribble complexes. The partitioning-defective (Par) complexes are comprised of Par3, Par6 and atypical protein kinase (aPKC), which localize to the TJ´s of the epithelial cell and play a key function in the assembly of the apical-lateral boundary. Additionally, Par3 can also localize to AJ´s and acts independently without forming a complex with the Par6 and aPKC (Morais-de-Sa et al., 2010).

The Crumbs complex consists of Crumbs homolog 3 (CRB3), Proteins associated with C.elegans Lin7 protein (Pals1) and Pals1 associated tight junction protein (PATJ) proteins. The Crumbs complex is also involved in defining the boundary between the apical and lateral domain (Medina et al., 2002). The Scribbled planar cell polarity protein (Scribble) complex consists of Scribble (SCRIB), Disc Large (DLG1-5), Lethal giant larvae (Lgl) proteins, Par1 and Par5, which are recruited to the basolateral domain of the cell. The main function of the Scribble protein complexes is to limiting the boundary of the apical domain (St Johnston and Sanson, 2011). Thus, the above mentioned protein complexes can interact to maintain the polarity in the epithelial layer.

Cytoskeleton controls cellular shape

As the name implies, the cytoskeleton forms the skeletal framework providing stability and structure to the cell. The cytoskeleton carries out numerous functions within the cell, but arguably the most important roles are: connecting the cell physically and biochemically to the external environment and stimuli, mediating forces to enable the movement and shape of the cell as well as the spatial organization of the cellular components. The cytoskeleton in eukaryotes consists of intermediate filaments, microtubules and actin filaments (Fletcher and Mullins, 2010). The primary function of these cytoskeletal components is to provide structure and shape to the cells, but they also differ in their physical properties when exposed to differing scenarios. Alterations in stiffness, changes in cytoskeletal regulation and assembly with different protein complexes can alter the function of the cytoskeleton and will therefore be further discussed in more detail in following sections of this thesis.

Microtubules are the largest and most rigid structure of the cytoskeleton and are composed of the globular tubulin proteins. Tubulin is classified into three types such as α, β and γ tubulin. The α and β – tubulins are organized in a polarized fashion with a rapid polymerizing plus end and a slower polymerizing minus end (Cooper, 2000b). The third subunit γ-tubulin plays an important role in microtubule assembly by localizing to the centrosomes, which are the initiation sites for microtubule organization. In non-polarized cells the minus end of the

microtubules are attached to the centrosome and the plus ends spread over the periphery of the cell. Upon initiation of polarization by cell-cell contacts the microtubules minus ends are released from the centrosome. Centrosomes are then arranged so that the minus ends face the apical side and plus ends face the basal side, which regulates the directional transport of polarity proteins to their respective domains in order to mediate cellular polarity (Cooper, 2000a; Muthuswamy and Xue, 2012). Formation of the mitotic spindle is also crucial for asymmetric and symmetric orientation during cell division (Bergstralh et al., 2013; Musch, 2004).

As indicated by their designation, the intermediate filaments are of intermediate size and are less rigid compared to microtubules and actin filaments. Intermediate filaments interact with actin and the microtubules to mediate the cell shape. Unlike the other two filaments, intermediate filaments are non-polarized and are composed of different proteins in a cell type specific manner. Prominent examples of intermediate filaments in differing cellular contexts are the keratin of epithelium, desmin in muscle cells, lamins in the nuclear envelope, α-internexin, neurofilament proteins in neuronal cells and vimentin within endothelial, mesenchymal and hematopoietic cells (Cooper, 2000a). One of the key functions of the intermediate filament is to reduce damage to the cell during mechanical stress, which will be discussed in further detail.

Despite being the smallest component of the cytoskeleton, actin filaments remain a major cytoskeletal constituent, consisting of 10% of total protein content in eukaryotic cells (Cooper, 2000a). Individual actin exists as globular actin (G-actin) monomers, which contain several binding sites where other actin monomers tightly bind and organize into higher order structures called actin filaments (F-actin). Actin filaments are polarized structures and are arranged in a head to tail fashion, consisting of a plus and minus end, similar to that of the microtubules structure (Winder and Ayscough, 2005). The formation of actin filaments is brought about by the polymerization of the monomeric actin subunits. Nucleation, the initial step of actin polymerization, is propagated when three actin monomers aggregate. The actin monomers are then added to both

ends resulting in the elongation of the actin filaments. During the elongation of the actin filament, actin monomers bind to ATP and are assembled in a bidirectional fashion. However, the binding rate of actin-ATP is relatively higher at the plus ends than the minus ends. The bound ATP can be readily hydrolyzed to ADP, which results in actin de-polymerization and prevents the elongation of the actin filaments by maintaining equilibrium between the actin monomers and actin filaments. This assembly and disassembly is regulated by the actin binding proteins (ABP). The actin related protein complex (Arp2/3), Profilin and Cofilin are three important ABPs, where Arp2/3 favors the nucleation of the actin filaments, Cofilin binds to the actin filaments and favors the disassembly of the filaments by hydrolyzing ATP to ADP, whereas Profilin favors the assembly of actin filaments by replacing ADP with ATP (Cooper, 2000a; Lodish H, 2000; Winder and Ayscough, 2005). Thus the organization and disorganization of the actin cytoskeleton are important in maintaining the cell shape, polarity, signal transduction, intracellular vesicular transport, cell attachment, cell division and cell motility (Heng and Koh, 2010).

Loss of tissue architecture in cancer

Cell migration is a crucial and highly organized process-taking place throughout the lifetime of an organism. During gastrulation the cells migrate to form the three germ layers ectoderm, endoderm and mesoderm. Cell sheets from the germ layers further migrate to the distant regions to form the specific organs. The migration mechanism is also very important in the adults to carry out vital function like maintaining the cell numbers, tissue repairing and also as a defense, where the immune cells migrate to distant places to patrol and to destroy the invaded pathogens (Heng and Koh, 2010; Ridley et al., 2003; Yamaguchi and Condeelis, 2007). This kind of migration requires transformation of epithelial cells to mesenchymal cells, which is referred to as epithelial to mesenchymal transition (EMT). This kind of conversion is reversible where the transformed epithelial cell regains its epithelial properties and is termed mesenchymal-epithelial transition (MET) (Kalluri and Weinberg, 2009). The EMT process is categorized into primary, secondary and tertiary EMT based on their function. Gastrulation, mammalian implantation and formation of the neural crest are

termed as primary EMT. Unlike the primary EMT the secondary EMT are found in wound healing, tissue regeneration and in organ fibrosis. The tertiary EMT occurs in cancer progression and metastasis. The importance of understanding the underlying mechanisms in EMT arises, since the analogous type of events like loss of cell-cell interaction, loss of polarity, cytoskeletal reorganization and increased motility of cells also occur during cancer metastasis (Kalluri and Weinberg, 2009; Thiery et al., 2009).

One of the initial steps during the EMT is the loss of E-cadherin, which is crucial for epithelial cell-cell interaction. The loss of E-cadherin is shown to be a sufficient event to transform a benign non-invasive adenoma into a malignant invasive carcinoma *in vivo* (Perl et al., 1998). The E-cadherin dysfunction can occur through germ line and somatic mutations, epigenetic modifications, transcriptional repression etc (Futreal et al., 2004; Jeanes et al., 2008). Moreover, a large number of growth factors like Transforming Growth Factor (TGF-β), Hepatocyte Growth Factor (HGF), Epidermal Growth Factor (EGF), Insulin-like-Growth Factor (IGF), Fibroblast Growth Factor (FGF) and their associated signaling pathways can regulate the loss of E-cadherin. As a result many transcriptional repressors of E-cadherin such as Snail, Zinc finger E-box-binding homeobox (ZEB), E47 and Twist are activated and repress E-cadherin expression (Yilmaz and Christofori, 2010). The loss of E-cadherin function in the tumor progression is accompanied by the increase of N-cadherin. This leads to loss of adhesive properties between and affinity for the neighboring cells, which is exploited by tumor cells to leave their tissue confinement and metastasize (Hulit et al., 2007; Nieman et al., 1999). The loss of apical-basal polarity in tumor progression is more complex and an important hallmark of EMT. The loss of the polarity protein Par3 is shown to induce metastasis in breast cancer through weakened cell-cell adhesion (Xue et al., 2013). Furthermore, studies in mice models also show that the deregulation of the Scribble protein complex can promote tumor progression (Pearson et al., 2011).

The relay of mechanical force is important for the formation and maintenance of tissue architecture

Tissues are continuously exposed to a plethora of intrinsic and extrinsic mechanical forces. Examples of extrinsic forces include gravity and exercise, which put particular stress on the tissues of the musculoskeletal system (Burkholder, 2007; Goldspink, 1999; Huijing and Jaspers, 2005; Rennie et al., 2004; Tidball, 2005). Intrinsic forces are more tissue specific, as different organs and cell types are exposed to differing degrees of mechanical forces. For example, the endothelial cells lining the aorta are physiologically exposed to higher shear stress than the endothelium of capillaries and thus the response of these cells to this stress is different (Chen, 2008). Cells exposed to mechanical forces are reliant on constant communication with neighboring cells and their surrounding ECM to respond to the external stimuli. Mechanotransduction describes the response of tissues and cells to mechanical stimuli and the translation of this mechanical force into biochemical signals, which influence cellular architecture and the regulation of genes (Paluch et al., 2015).

The contraction of cells generates mechanical forces, which can influence tissue architecture. This has been shown to be vital in development, where the formation of certain structures such as cavities and folds during embryogenesis is reliant on such forces (Keller et al., 2003; Kiehart et al., 2000; Odell et al., 1981). Mechanical forces also influence proliferation, differentiation and migration of cells during development as well as intracellular processes such as signal transduction and gene expression (Farge, 2003; Keller et al., 2008; Lee et al., 2006; Somogyi and Rorth, 2004; Tan and Katsanis, 2009). Thus, mechanical forces are indispensable in the control and development of functional tissues, organs and their homeostasis.

Although required for regulation and development of organs, aberrant mechanical forces can result in disease (Chen, 2008; Krieg et al., 2008; Lo et al., 2004; Sordella et al., 2003). Examples of pathological conditions that arise from defective mechanotransduction include hearing loss, atherosclerosis, muscular dystrophy, asthma, osteoporosis, polycystic kidney disease and cancer (Affonce and Lutchen, 2006; Gulino-Debrac, 2013; Hammerschmidt et al., 2007;

Heydemann and McNally, 2007; Jaalouk and Lammerding, 2009; Vollrath et al., 2007). In order to understand mechanotransduction in more detail, it is important to consider the sites where mechanical forces can modulate cellular processes. In eukaryotic cells, mechanical forces act upon cell-cell and cell-ECM contacts and relay signals intra-cellularly to modulate the cytoskeleton of individual cells.

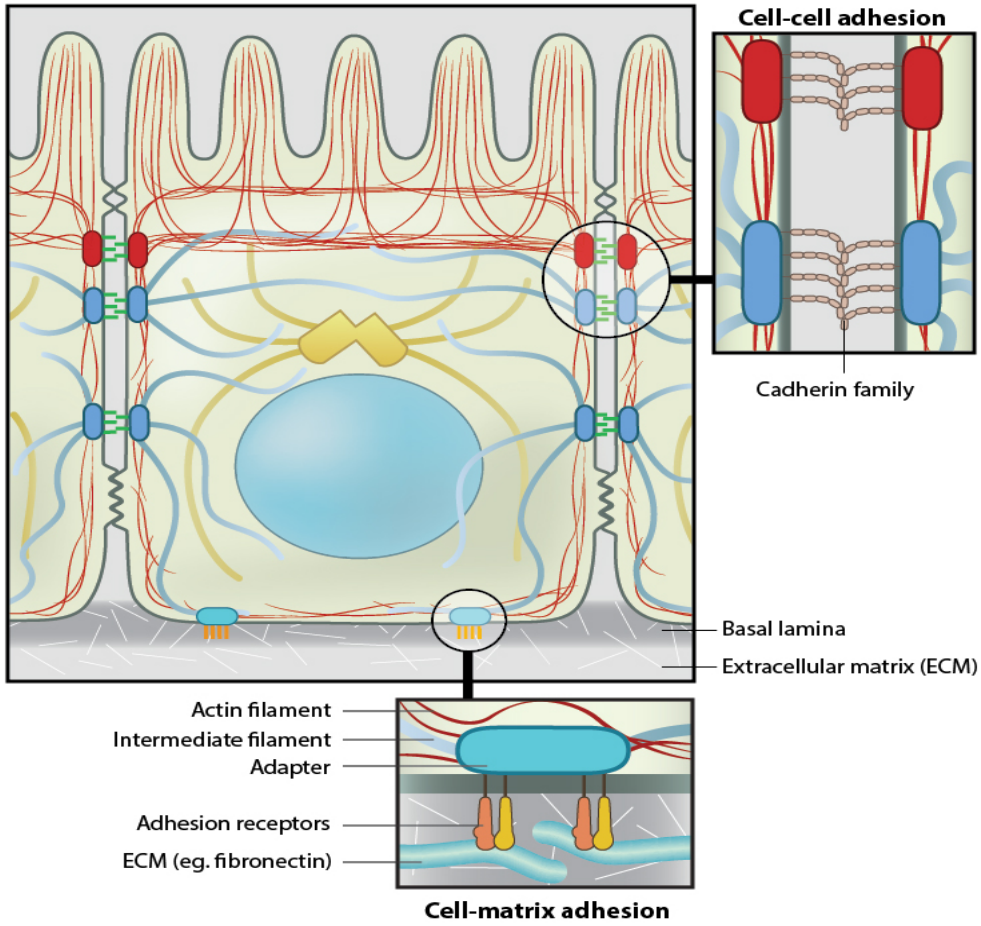

Figure 3: Cell adhesion receptors: Cell-cell adhesion receptors (eg, Cadherin transmembrane proteins) connecting the adjacent cells, actin cytoskeleton and cell-Matrix adhesion receptors (eg, Integrins) connecting the ECM and actin cytoskeleton within the cell. The cell adhesion receptor enables the transmission and sensing of mechanical forces via their connection to the actin cytoskeleton. Adapted from https://www.mechanobio.info and licensed by creativecommons.org/licenses/by/4.0/

Mechanotransduction at cell-cell contacts

The cadherins were recently identified as bonafide transmitters of mechanical signals between cells. Cadherins also act as mechanosensors and adapt to physical stress generated by altering the stiffness of the ECM (Ganz et al., 2006; Ladoux et al., 2010; Takeichi, 2014). The classical cadherins such as Vascular Endothelial (VE) cadherin of endothelial cells and E-cadherin of epithelial cells form homophilic complexes with neighboring cells, which is mediated by their extracellular domain. The intracellular C-terminal domain of cadherins binds to β-catenin, which in turn binds to α-catenin, an adaptor protein that facilitates binding with F-actin filaments and Vinculin (le Duc et al., 2010). Vinculin, together with other AJ´s proteins, forms the mechanical connection between the cell-cell adhesion proteins and the contractile actomyosin cytoskeleton (le Duc et al., 2010; Pokutta and Weis, 2007; Watabe-Uchida et al., 1998). Furthermore, the association of vinculin with α-catenin is strictly regulated by force generated by the actin cytoskeleton. Contractile forces generated by neighboring cells induce a conformational change in α-catenin, exposing the vinculin-binding site and facilitating the association of Vinvulin (Yonemura et al., 2010). The α-catenin-Vinculin connection is essential for the maintenance of cell-cell connections and resistance to mechanical forces in tissues (Ladoux et al., 2010; le Duc et al., 2010; Leerberg et al., 2014). These findings collectively show a role for cadherins at the cell-cell contacts as receivers and transmitters of mechanical forces.

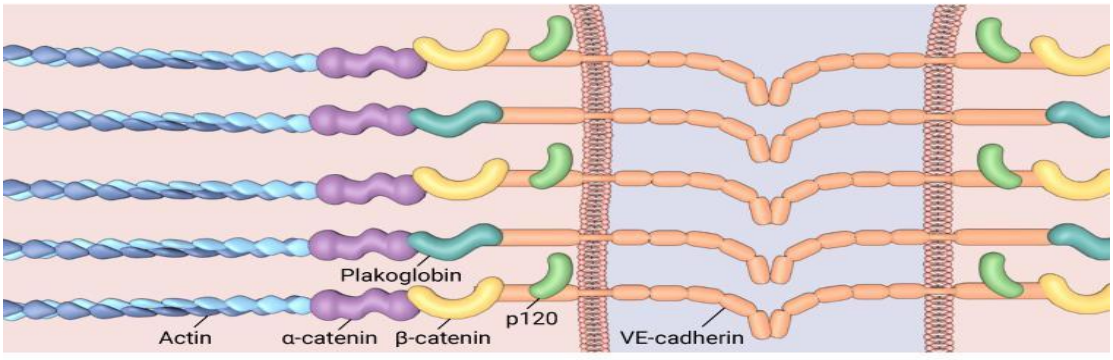

Figure 4: Cell-cell Adhesome: Adherens Junctional complex comprised of cadherin transmembrane proteins connecting adjacent cells; intracellulary cadherins are connected to α- and β-catenins, which in turn are linked to the actin cytoskeleton, thus enabling mechanotransduction from AJ´s. Adapted from https://www.mechanobio.info and licensed by creativecommons.org/licenses/by/4.0/

Mechanotransduction by cell-ECM contacts

The cell receptor proteins and their respective ligands found in the ECM form the initial interactions between the cell and the ECM. Of these receptors, integrins have been identified as a major family of cell adhesion receptors, which bind to the ECM (Alenghat and Ingber, 2002; Burridge and Chrzanowska-Wodnicka, 1996; Katsumi et al., 2004). In particular, integrins have been shown to bind to collagen and fibronectin, which are key components of the ECM. These interactions between the cell and the ECM are referred as "Focal Adhesions" which can span up to several microns in area, indicating their importance in anchoring the cell to the ECM (Doyle and Yamada, 2016; Geiger et al., 2001; Humphries et al., 2006; Schwartz, 2010). The intracellular region of the integrin receptor connects to the actomyosin cytoskeleton, thereby providing a mechanical connection between the cell and the ECM (Harburger and Calderwood, 2009; Hynes, 2002). More than 150 proteins have been identified, which interact either directly or indirectly with the actin cytoskeleton and cytoplasmic region of integrins mediating focal adhesion formation (Harburger and Calderwood, 2009; Hynes, 2002; Lo, 2006; Zaidel-Bar et al., 2007). The key constituent proteins mediating this interaction are Talin, Paxillin, Vinculin, α-actinin, Focal Adhesion kinase (FAK), Src, Zyxin, Filamin and Tensin (Bershadsky et al., 2006; Geiger et al., 2001). The associations of these proteins to the cytoplasmic domain of integrins are regulated by the stiffness of the ECM and contractile forces generated by the actin cytoskeleton (Bershadsky et al., 2006). For example, force-induced conformational change of Talin exposes a Vinculin-binding site, allowing Vinculin to bind to the actin cytoskeleton and induce cytoskeletal stiffening (Spanjaard and de Rooij, 2013). Thus, the above findings strongly emphasize integrin mediated adhesion to the ECM as a key signaling hub for mechanotransduction, which mediates inside-out signaling to interpret external stimuli (Calderwood et al., 2000; Evans and Calderwood, 2007).

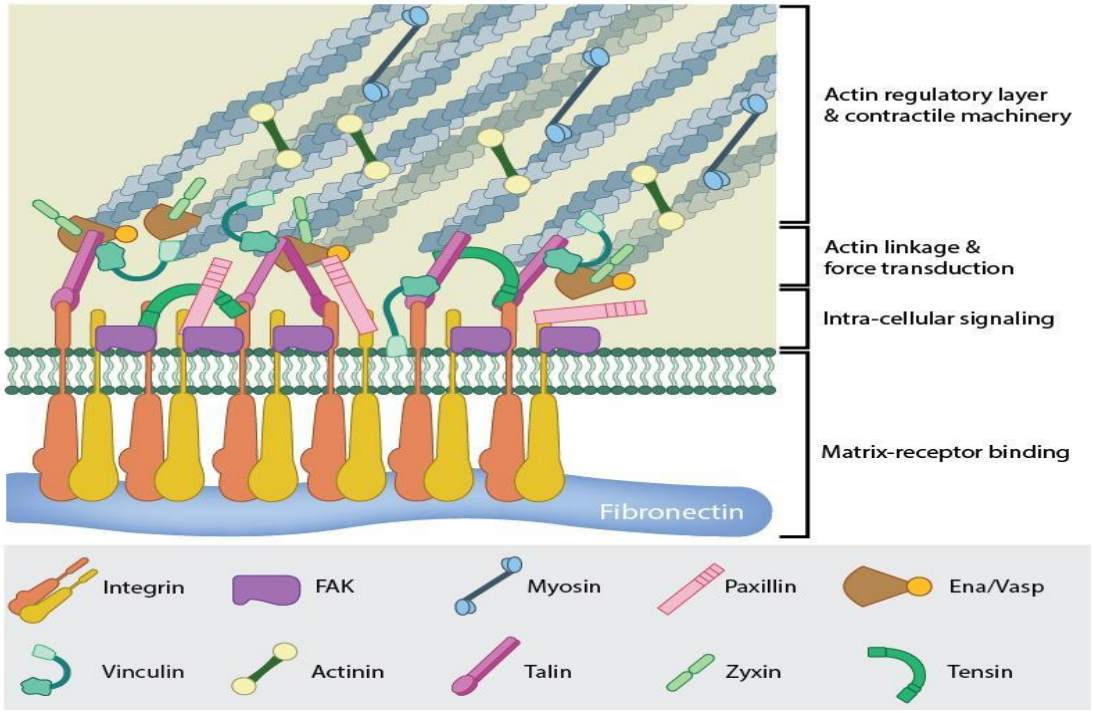

Figure 5: Focal Adhesion complex: Representative image of a mature Focal Adhesion (FA) comprised of a plethora of interacting proteins enabling cell-ECM binding, linkage to the actin cytoskeleton and transmission of mechanical forces. Adapted from https://www.mechanobio.info and licensed by creativecommons.org/licenses/by/4.0/

The actin-cytoskeleton transduces mechanical forces inside the cell

The cytoskeleton plays a central role in responding to mechanical cues generated from both the cell-cell and cell-ECM contacts (Geiger et al., 2001; Ohashi et al., 2017; Perez-Moreno et al., 2003). Actin is a well-studied component of the cytoskeletal proteins and is associated with a majority of cellular feedback mechanisms. These range from biochemical to mechanical and result in altered cell shape, cell motility, intracellular trafficking and force generation (Fletcher and Mullins, 2010). Recent advancements in high resolution imaging techniques allow more detailed analysis, which indicated that the actin cytoskeleton is assembled into various unique higher order structures including filopodia, lamellipodia, stress fibers, cell cortex, podosomes, actin asters and stars (Fritzsche et al., 2017; Harris et al., 2018; Michelot and Drubin, 2011; Wang,

2017). Several studies utilizing electron microscopy, Forster Resonance Energy Transfer (FRET) probes, optical tweezers, optical traps and Atomic Force microscopy (AFM) also elucidated that specialized actin structures exhibit a mechanical tension ranging from 1 pN to 100 pN (Harris et al., 2018; Wang and Kanchanawong, 2016). The polymorphic arrangement of the actin cytoskeleton within a single cell indicates the dynamic nature of the actin filaments and ABP, which readily undergo remodeling upon exposure to external stimuli (Harris et al., 2018; Shimozawa and Ishiwata, 2009; Wang, 2017). Upon exposure to mechanical stress, actin filaments either polymerize or depolymerize to increase or decrease the filament length. The actin filament regulatory proteins; Arp2/3 (branched actin nucleation) and cofilin (actin severing) alter actin filaments in a tension dependent manner (Hayakawa et al., 2014; Hayakawa et al., 2011; McGough et al., 1997; Risca et al., 2012; Uyeda et al., 2011). Similarly to actin filaments, mechanical signals induce conformational changes in ABP´s, well-known examples for this are α-catenin in the AJ, Vinculin and Talin in the FA (Buckley et al., 2014; del Rio et al., 2009).

Furthermore, mechanical signals were also speculated to change the kinetics of actin polymerization by ABP´s in order to regulate the actin cytoskeletal density and growth rate. Profilin, another ABP, regulates actin polymerization kinetics by associating with the actin nucleating protein formin (Goode and Eck, 2007). *In vitro* studies showed that recruitment of profilin accelerates the actin filament polymerization, whereas the absence of profilin greatly reduced actin filament polymerization (Courtemanche et al., 2013; Higashida et al., 2013; Kubota et al., 2017). Collectively, these data prove that the actin cytoskeleton acts as a mechanosensor by rapidly altering its composition, organization and function to enable cells to sense and adapt to their immediate environment.

The cellular nucleus responds to external mechanical forces

Mechanical forces, which act upon the cadherins and integrins, are relayed over long distances via the cytoskeleton and eventually converge on the nucleus (Maniotis et al., 1997; Wang and Suo, 2005; Wang et al., 2009). The link between the nucleus and cell-cell contacts or FA`s is either direct or indirect and is

mediated by a number of adaptor proteins (Boban et al., 2010; Geiger et al., 2001; Wang et al., 2009). The Klarsicht, ANC-1, and Syne Homology (KASH) domain proteins, which include nesprins, are localized on the outer nuclear membrane (ONM) and connect the cytoskeleton to the nucleus. The C-terminal domain of nesprin binds to Sad1, UNC84 (SUN) domain proteins in the inner nuclear membrane (INM), where the SUN proteins are internally anchored to the nuclear lamina and chromatin (Furukawa et al., 2009). The association of cytoskeletal networks with nesprins in ONM and SUN domain proteins in INM forms a larger signaling complex known as Linker of Nucleoskeleton and cytoskeleton (LINC) complex (Alam et al., 2015; Crisp and Burke, 2008; Jahed et al., 2014; Schirmer and Foisner, 2007). Collectively, this shows that the nucleus is mechanically tethered to the cell surface receptors via the LINC complex. Importantly, this tethering enables the nucleus to respond to its surroundings and adapt accordingly by altering the nuclear morphology. Previous work has shown that the nuclear morphology can be modulated by the stiffness of the underlying ECM. Cells, which were plated onto a stiff surface, exhibited a flattened nuclear morphology, whereas softer matrices resulted in a more rounded nuclear morphology (Lovett et al., 2013). Apart from maintaining nuclear morphology, the LINC complex is essential for proper nuclear positioning and regulating cellular processes such as polarization, differentiation, migration and cellular division (Holaska et al., 2004; Pederson and Aebi, 2002).

As the nucleus forms one of the largest organelles in the cell, it must be able to withstand incoming mechanical stress in order to preserve its contents; genetic material. Several studies emphasize that, depending on the cell type, the nuclear stiffness can vary from 3-10 times higher than that of the cytoplasm. Lamins, proteins found within the nuclear envelope, are responsible for the maintenance of nuclear stiffness and nuclear morphology (Lammerding et al., 2006; Pajerowski et al., 2007). Lamin A and C are the main constituent proteins found to regulate the nuclear stiffness and shape by forming a network of intermediate filaments beneath the INM described as the “Nuclear Lamina” (Lammerding et al., 2006; Pajerowski et al., 2007; Schape et al., 2009). Furthermore, mutations in

Lamin A/C have been found to be associated with certain pathologies including cancer, where these mutations potentiate cancer cell migration through tight spaces within tissues as the nucleus becomes more malleable (Hatch et al., 2013; Vargas et al., 2012). In fact, mutations in any of the connecting partners including the cytoskeleton and LINC complex were shown to affect nuclear morphology and integrity (Stroud, 2018). Additionally, mechanical forces have also been shown to play a role in regulating gene transcription (Jain et al., 2013; Makhija et al., 2016; Uhler and Shivashankar, 2017). These findings highlight the importance of a number of regulatory complexes in mediating mechanotransduction from the cell surface to the nucleus and that these are crucial in maintaining proper nuclear function and ultimately decisions of cell fate.

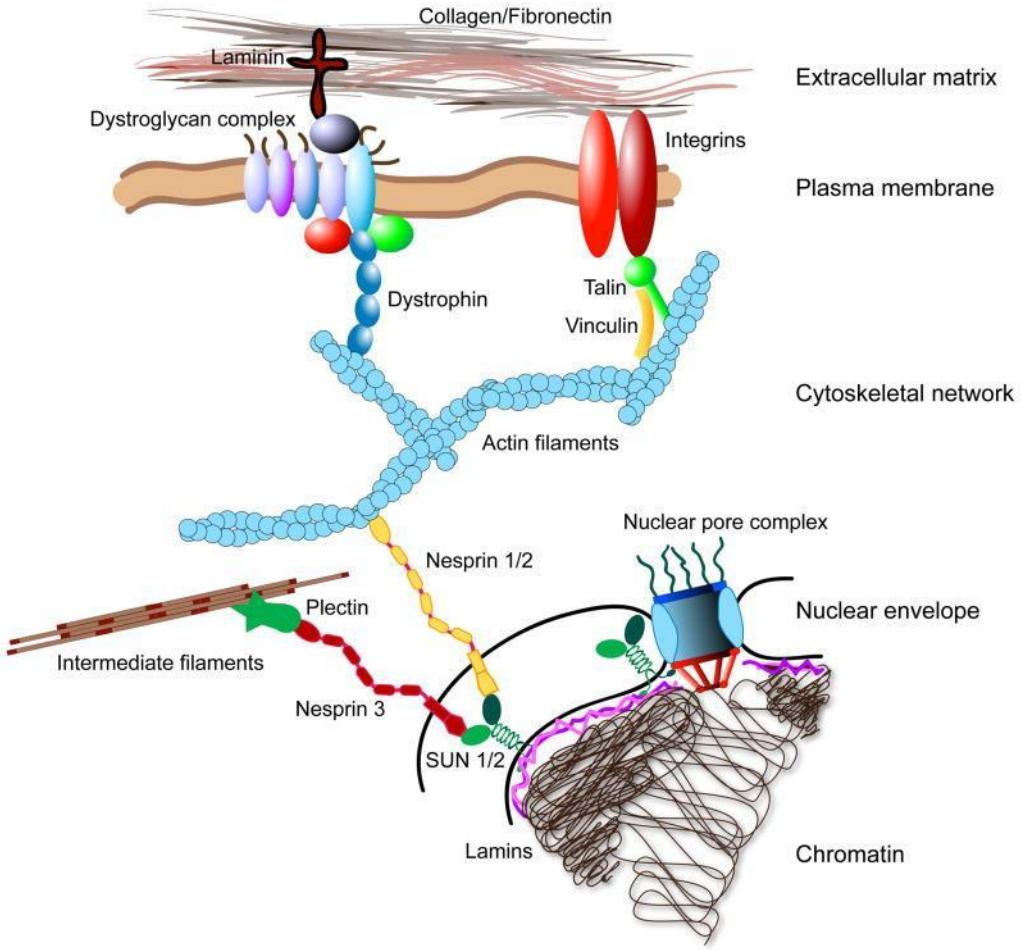

Figure 6: Force transmission from FA to Nucleus: Integrins and other adhesion molecules couple the ECM to the actin cytoskeleton. The actin cytoskeleton can in turn bind to the nuclear

envelope via nesprins in the ONM. At the INM, nesprins bind to SUN domain proteins, thereby creating a physical link from FA to the nucleus. Adapted from (Jaalouk and Lammerding, 2009).

Mechanotransduction through the HIPPO signaling pathway

The HIPPO signaling pathway is a highly conserved pathway and regulates a number of processes, including cellular proliferation, differentiation and apoptosis in order to control organ growth and size (Verma et al., 2018; Zeng and Hong, 2008; Zhao et al., 2010). The HIPPO pathway was first identified in *Drosophila* and is comprised of two key transcriptional co-activators, Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ) (Justice et al., 1995; Xu et al., 1995). YAP was first identified by Marius Sudol, whilst studying the potential binding partners of the SH3 domain of the protooncogene c-Yes (Zhao et al., 1990). Further studies of the YAP gene revealed two closely related binding motifs; WW and PPxY domains consisting of 38 amino acids (Bork and Sudol, 1994; Sudol et al., 1995a; Sudol et al., 1995b). The WW domain and its analogous PPxY motif facilitate YAP's association with the proline rich motifs of other members in the HIPPO signaling cascade (Chen and Sudol, 1995; Sudol and Harvey, 2010). Various stimuli including contact inhibited growth have been shown to trigger signaling proteins upstream of YAP/TAZ. For example, the MST1/2 protein kinases, which phosphorylate the downstream LATS1/2 serine/threonine kinases, have been shown to be activated by contact inhibition (Chan et al., 2005; Wu et al., 2003; Zhao et al., 2007). Activated LATS1/2 phosphorylates YAP/TAZ and retains it within the cytoplasm through interactions with 14-3-3 proteins, hence inhibiting the nuclear localization of YAP/TAZ (Dong et al., 2007; Kanai et al., 2000; Oka et al., 2008; Sudol, 2010). Consequently, YAP/TAZ co-activators are unable to initiate transcription of their target genes. This control of YAP/TAZ activity allows cells to tune their proliferative rate to control organ growth and size accordingly.

In addition to contact inhibited growth, YAP activity is also modulated by mechanical stressors. *In vitro* studies have shown that YAP is phosphorylated and localized in the cytoplasm when cells are grown to confluency. Conversely, in sub-confluent cells, YAP translocates to the nucleus enabling concomitant

transcription of target genes (Zhao et al., 2007). E-cadherin associates with α- and β-catenin at cell-cell junctions, an interaction, which is also speculated to drive cell-cell contact inhibition of YAP activity (Kim et al., 2011). Interestingly, when contact inhibited cells were mechanically stretched, YAP/TAZ was found to translocate from the cytoplasm to the nucleus and trigger cell proliferation (Aragona et al., 2013). This raises further questions; such as whether stretching of the basal membrane of confluent cells would also influence YAP activity. Recently, several studies have implicated cell-ECM interactions in the regulation of YAP. One such study revealed that when cells are plated on microfabricated adhesive patterns of varying area and size, YAP accumulates in the nucleus of cells cultured in large domains whereas in those in the small domains YAP is localized in the cytoplasm (Wada et al., 2011).

Mechanical cues transmitted from cell surface receptors are channeled via the cytoskeleton. Interestingly, F-actin has been shown to mediate the response to matrices stiffness and the regulation of YAP (Low et al., 2014; Sansores-Garcia et al., 2011). F-actin (stress fibers induced by ECM) was shown to modulate phosphorylation of YAP by modulation of upstream LATS kinase; as a result YAP is sequestered to the cytoplasm (Wada et al., 2011). Conversely, blocking of actin polymerisation using inhibitors such as lantranculin A, cytochalasin D and blebbistatin inhibited the stiffness-induced nuclear translocation of YAP (Wada et al., 2011). This suggests an important role for mechanical stress at the cell-cell and ECM contacts in the regulation of YAP activity. Indeed, these findings are crucial in understanding how altered stiffness of the ECM could regulate the Hippo signaling pathway and contribute to tumor progression, as the tumor stroma has been shown to exhibit altered stiffness.

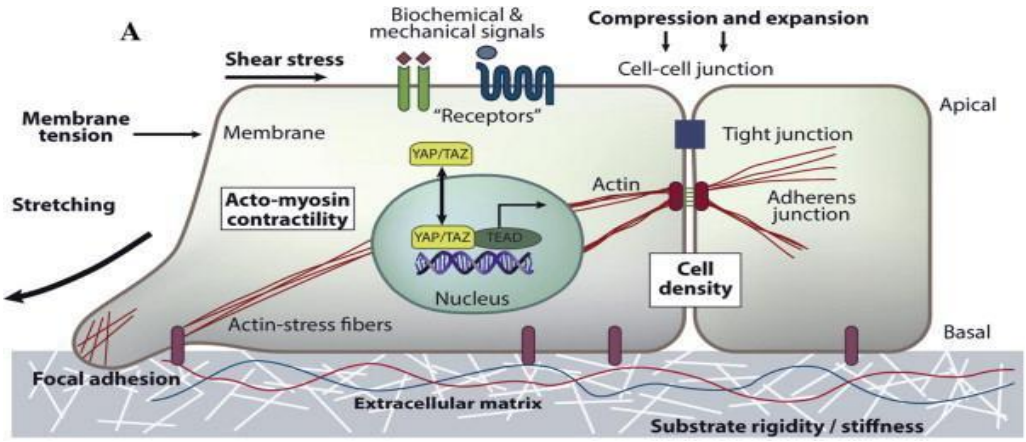

Figure 7: YAP/TAZ in mechanotransduction: Mechanical cues from cell adhesion receptors channeled via the actin cytoskeleton are essential for YAP/TAZ regulation. Upon activation YAP/TAZ translocates from the cytoplasm to the nucleus and associates with TEAD transcription factor, thus initiating transcription of target genes and ultimately enabling cell proliferation. Adapted from (Low et al., 2014)

Mechanotransduction in tumor progression

It is evident that communication between the cell and its surrounding microenvironment is important for vital biological processes such as proliferation, differentiation, migration and homeostasis. Furthermore, recent advancements in the field of mechanobiology have elucidated the role of cellular mechanics and disease progression. Alterations in canonical mechanotransduction or mutations in mechanosensory proteins are associated with a number of disease states including cancer (Jaalouk and Lammerding, 2009). Solid tumors are generally stiffer than their surrounding tissue. Extensive research over the past decade has shown that radical changes in ECM stiffness, remodeling, and the resultant cytoskeletal disorder promotes malignant transformation, tumor progression and metastasis (Huang and Ingber, 2005; Makale, 2007; Suresh, 2007; Wolf et al., 2007).

In breast cancer progression, increased ECM rigidity was shown to initiate the differentiation and proliferation of mammary epithelial cancer cells (Bissell et al., 2005; Huang and Ingber, 2005; Weaver et al., 1997). The ECM stiffness may emanate from fibrosis or increased cytoskeletal tension caused by oncogenic

(Ras) mediated extracellular signal-regulated kinases (ERK) activation (Huang and Ingber, 2005). Furthermore, ECM stiffness has been shown to potentiate tumor development and progression, as the FAs of cancer cells respond to ECM stiffness by activating the Rho and Rho associated Coiled-coil containing protein kinase (ROCK) pathway (Hebner et al., 2008). The activation of ROCK accelerates phosphorylation of myosin light chain (MLP) resulting in increased cytoskeletal tension, which creates a positive feedback loop creating greater ECM stiffness via inside-out mechanical signaling (Gaggioli et al., 2007). Inter-play between the Rho-ROCK pathway and the Epidermal Growth Factor (EGFR)-Ras-ERK pathway results in higher proliferation rates in mammary epithelial cancer cells (Wei et al., 2016). Alterations in the physical environment (ECM stiffness) of tumors has also been shown to affect apical–basal polarity and initiate the invasion of cells into the cystic lumen of mammary ducts, commonly referred to as Ductal Carcinoma In-situ (DCIS) (Jaalouk and Lammerding, 2009; Paszek et al., 2005; Schedin and Keely, 2011).

Due to the continual unchecked proliferation of cancer cells, tumors experience increased mechanical pressure compared to surrounding healthy tissues. This mechanical pressure alters the tumor cell cytoplasm via the actin cytoskeleton and influences overall cell and nuclear mechanics (Lele and Kumar, 2007; Makale, 2007; Wyckoff et al., 2006). Consequently, tumor cells exhibit altered cyto-adherence and gene expression, which facilitates their survival and further promotes invasion (Makale, 2007). Indeed, the ability of tumor cells to invade the surrounding ECM and small interstitial pores relies heavily on their ability to deform their cellular structures.

The Angiomotin protein family

The Angiomotin protein family consists of three members, Angiomotin (Amot), Angiomotin Like 1 (AmotL1) and Angiomotin Like 2 (AmotL2). These scaffold proteins are found in human, mice and zebrafish. However, there is no orthologous Angiomotin gene found in Drosophila, bacteria or yeast genomes (Bratt et al., 2002). The family of Angiomotin protein contain several binding sites, which allow the Angiomotins to influence multiple cellular processes such

as polarity, cell-cell interaction and cytoskeletal organization. Amot was the first protein to be identified in this family through screening of angiostatin binding peptides in a yeast two-hybrid cDNA library using constructs containing kringle domains (1-4) of angiostatin. Subsequent studies have gone on to show that Amot regulates cell migration and tube formation of endothelial cells (Troyanovsky et al., 2001). The two other members of the angiomotin family AmotL1 and AmotL2 were identified by screening of TJ proteins and homology searches in GenBank (Bratt et al., 2002; Nishimura et al., 2002). A common feature of each member of the Angiomotin family is that, they all have two isoforms; consisting of a full length and a shorter variant. The full-length isoforms of the Angiomotins consist of an N-terminal domain whereas the shorter isoforms lack such a domain. The N-terminus of the full-length isoforms contains a glutamine rich domain, LPxY and PPxY motifs, which facilitate interactions with MAGI-1, YAP and NEED4 (Patrie, 2005; Wang et al., 2012). Additionally, the Angiomotin family contains a coiled-coiled domain, which is highly conserved in both the longer and shorter isoforms of the family. The coiled-coil domain facilitates the interaction of the Angiomotins with a number of other signaling proteins. For example, the coiled-coil domain of Amot has been shown to associate with Merlin, a tumor suppressor, and disconnect Rich1 thereby inhibiting Rac and Ras activity (Ernkvist et al., 2008; Zheng et al., 2009). The coiled-coil domain also functions as a site of oligomerisation allowing Angiomotin family members to associate and form homo- and hetero-oligomers (Ernkvist et al., 2008; Patrie, 2005; Wang et al., 2012; Zheng et al., 2009). Angiomotin proteins also play an important role in regulating polarity through association with the partitioning defective 3 homolog (Par3) and crumbs homolog 3 (CRB3) proteins through their C-terminal PDZ domain. The PDZ binding motif of Amot has also been shown to be essential for the migration of endothelial cells, which is mediated by an association with Syx, a Rho-GTPase exchange factor (Ernkvist et al., 2009; Wells et al., 2006). Furthermore, Amot associate with actin filaments in epithelial cells to promote remodeling of the actin cytoskeleton (Ernkvist et al., 2006). Additionally, depletion of Amot in mouse embryos induced early lethality due to defects in migration, which is vital for gastrulation (Shimono and Behringer, 2003). Interestingly, it has been

recently shown that Amot interacts with members of the HIPPO signaling pathway (Paramasivam et al., 2011). Angiomotins have been shown to associate with YAP and negatively regulate the HIPPO pathway by retaining YAP within the cytoplasm (Paramasivam et al., 2011; Wang et al., 2012; Zhao et al., 2011).

The second member of the angiomotin family, AmotL1, also binds to actin filaments and promotes cytoskeletal organization, which has been shown to be important for the junctional stability of the tip cells during angiogenesis (Gagne et al., 2009; Zheng et al., 2009). The final member of the angiomotin family AmotL2 exists as two isoforms: a longer p100AmotL2 and shorter p60AmotL2. It was initially suggested that p100AmotL2 associate with the junctional protein MAGI-1 through its N-terminal domain (Patrie, 2005). Further studies have revealed that AmotL2 is important for cell migration during gastrulation in zebrafish embryos in response to the fibroblast growth factor (FGF) signaling, where the inhibition of AmotL2 using antisense morpholinos causes the arrest of epiboly (Huang et al., 2007). Recently published work from our lab further explored the role of AmotL2 in vascular development and tumor progression. *In vitro* studies in mouse endothelial cells showed that AmotL2 acts as a linker between VE-cadherin and the actin cytoskeleton (Hultin et al., 2014). AmotL2 has also been shown to be crucial for aortic lumen expansion in zebrafish and mice, highlighting its importance in the maintenance of tissue architecture. Depletion of AmotL2 by morpholino injection in zebrafish and genetic deletion in mice exhibited collapsing of the dorsal aorta (Hultin et al., 2014). The shorter p60 isoform of AmotL2 expression is responsive to hypoxia, where expression levels increased after 3h and 8h exposure to hypoxia *in vitro* (Liu et al., 2007; Mondon et al., 2005). In addition to this, AmotL2 expression was shown to co-localize with the hypoxic markers CA9 in human colon cancer. Furthermore, p60Amotl2 traps and sequesters the polarity proteins (Par3, Crb3) in intracellular vesicles, thereby inhibiting their transport to the cell junctions (Mojallal et al., 2014). As already alluded to, Amotl2 has two isoforms, a short p60 variant and a full-length p100 isoform. The p60 AmotL2 was shown to influence invasion of cancer cells, as p60AmotL2-expressing Caco2 cells injected into mice invaded the surrounding stroma, suggesting a role in tumor

progression and metastasis (Mojallal et al., 2014). In this thesis we have reported a connection between AmotL2 and E-cadherin/catenin complex. Additionally, we have shown that AmotL2 association with the above complex is required for the organization of radial actin filaments, regulating endothelial and epithelial cell morphology. We have also elucidated that the above connection is directly coupled to the nuclear envelope and enables the nucleus to sense the incoming mechanical forces from cell junctions. Interestingly, the shorter p60AmotL2 isoform disrupts the above mechanotransductory complex, resulting in altered cell-cell cohesion and nuclear morphology promoting single cell migration, when sensitized to growth factor signaling.

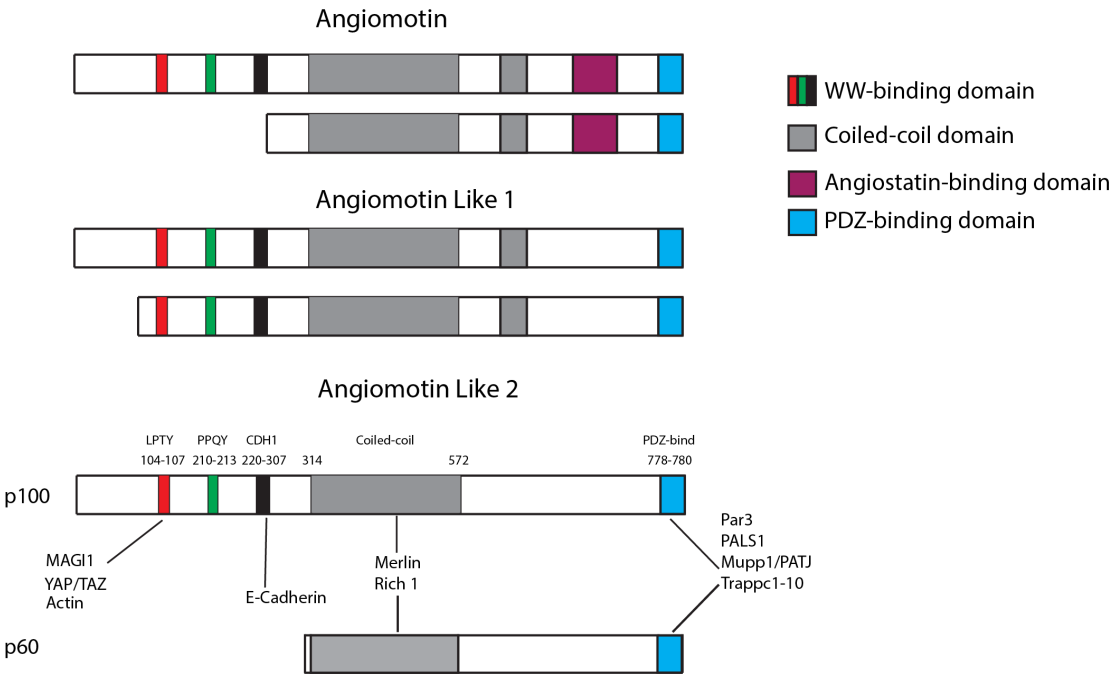

Figure 8: The Angiomotin protein family: The Angiomotin protein family consists of three members. Each member has a longer and shorter isoform, Amot (p130 & p80), AmotL1 (p100 & p90) and AmotL2 (p100 & p60). The primary domain structure of AmotL2 including the binding sites and interaction partners are mentioned above.

Aims

The overall aims of this thesis were to investigate the role of the E-cadherin/AmotL2 complex during normal development and cancer progression.

Paper 1

To investigate how AmotL2 and the polarity protein Par3 controls aortic expansion in zebrafish as well as the geometrical packing of epithelial cells.

Paper 2

To investigate the role of AmotL2 as a novel component of the E-cadherin complex and how this complex affects cytoskeletal function *in vitro* and *in vivo.*

Paper 3

To investigate whether the stress-induced p60AmotL2 isoform may act as a dominant-negative and thereby control epithelial integrity.

Paper 4

To explore the normal function of p60AmotL2 in epithelial homeostasis.

Results and discussions

Paper 1

AmotL2 integrates polarity and junctional cues to modulate cell shape

The overall aim of the study presented in this paper was to analyze the importance of Par3 in localizing p100AmotL2 to the cell-cell junctions. We have previously shown that AmotL2 localizes to the cell-cell junctions and associates to the VE- and E-cadherin protein complex and is crucial for actin filament organization, maintaining cellular geometry and cell packing (Hildebrand et al., 2017; Hultin et al., 2014). We have also previously reported that AmotL2 binds directly to the polarity protein Par3 through its C-terminal PDZ binding domain (Hultin et al., 2014). Thus, AmotL2 acts as a scaffold protein and integrates the junctional, cytoskeletal and polarity cues required for organ formation in the development of the dorsal aorta and blastocyst hatching (Hildebrand et al., 2017; Hultin et al., 2014).

Par3 controls dorsal aorta development in zebrafish by transporting AmotL2 to cell-cell junctions

The Par3 polarity protein was earlier reported to be essential for apical-basal polarization and lumen formation in both epithelial and endothelial cells (Denker et al., 2013; Jaffe et al., 2008; Koh et al., 2008; Martin-Belmonte et al., 2007). Additionally, Par3 was reported to be crucial for aortic lumen formation in mice, where Par3 expression rescued the aortic defects exhibited by β1-integrin depletion (Zovein et al., 2010). In our recent study we showed that AmotL2 is essential for the lumenization of the dorsal aorta in zebrafish (Hultin et al., 2014). Furthermore, AmotL2 binds to Par3 through the C-terminal PDZ binding domain (Hultin et al., 2014). This suggests that Par3 and AmotL2 are in the same pathway. To analyze the role of Par3 in aortic development, we silenced Par3 in zebrafish using anti-sense morpholino oligos (MO). The dosage of MO were carefully optimized so as to visualize the vascular defects, as previous studies reported that a higher dosage of Par3 MO caused severe brain and eye defects in zebrafish (Wei et al., 2004). The efficiency of Par3 MO was

analyzed by western blot. At 48h post fertilization, Par3 MO embryos suffered brain and pericardial edema. When closely analyzed using the double transgenic model Tg(gata1:dsRed)^{sd2} and Tg(kdrl:EGFP), we found the Par3 morphants lacked functional blood circulation and dsRed erythrocytes accumulated in the pericardium. In contrast, the erythrocytes of the control embryos showed normal circulation within EGFP positive vascular networks. Silencing of Par3 expression affected the dorsal aorta, which exhibited constrictions whereas the pericardial vein remained lumenized. Interestingly, a similar phenotype was previously observed when AmotL2 was silenced using anti-sense MO in zebrafish embryos (Hultin et al., 2014).

Par3 indirectly controls actin filament organization through AmotL2 in zebrafish skin

AmotL2 mediates the connection between junctional proteins VE- and E-cadherin to radial actin filaments in endothelial and epithelial cells. We have reported this connection is crucial for controlling the cell shape and cell packing (Hildebrand et al., 2017; Hultin et al., 2014). We have also shown that AmotL2 depletion resulted in disruption of actin filaments organization, altered epithelial geometry and packing in the dermis of zebrafish skin (Hildebrand et al., 2017). To analyze the effect of Par3 silencing in the zebrafish dermis, we injected Par3 MO into zebrafish embryos. Immunofluorescent microscopy showed disrupted actin filaments and altered cellular geometry with increased cell area in Par3 depleted fish. AmotL2 localization to cell-cell junction also showed a significant decrease. Co-injection with human Par3 mRNA rescued the cellular geometry, which was evident with a decrease in cell area. Thus, Par3 MO strongly mimicked the phenotype of AmotL2 morphants in zebrafish skin.

AmotL2/Par3 protein complex induces actin filament organization *in vitro*

Immunofluorescent microscopy of Bovine Capillary Endothelial cells (BCE) showed localization of AmotL2 and Par3 at cell-cell junctions. This was further analyzed by co-immunoprecipitation using an AmotL2 antibody, where AmotL2 pulled down both Par3 and Par6 together with aPKC. This was expected as

previous findings indicate that Par3 complexes with Par6 and aPKC in the apical junction of cells (Iden et al., 2006; Izumi et al., 1998; Macara, 2004; Tepass, 2012; Wodarz et al., 2000). Additionally, immunostaining of Mile Sven-1 (MS-1) mouse pancreatic islet endothelial cells indicated that silencing of Par3 by siRNA resulted in disruption of radial actin filaments in a similar fashion to AmotL2 siRNA. From the above results we speculated that Par3, together with AmotL2, control radial actin filament organization.

Par3 facilitates the transport of AmotL2 to adherens junctions

To further analyze the role of Par3 in AmotL2 localization we depleted both Par3 and AmotL2 using siRNA in Ms-1 cells. Interestingly, we found that reducing Par3 levels disrupted the localization of AmotL2 to the cell-cell junctions, whereas localization of β-catenin to the cell-cell junctions was unaffected. However, siRNA depletion of AmotL2 did not disrupt the junctional localization of Par3, as analyzed by the overlap with β-catenin. These results were in line with the previous findings indicating that Par3 associates with β-catenin at cell-cell junctions (Wei et al., 2005). Analysis by western blot showed that silencing of AmotL2 does not affect the total protein levels of Par3 in Ms-1 cells, arguing for a specific role of Par3 in transporting of AmotL2 to AJ. Furthermore, injection of human Par3 mRNA alone or in combination with AmotL2 mRNA in zebrafish skin resulted in Par3 localization to cell junctions irrespective of AmotL2 localization.

Par3 is essential for AmotL2 association with E-cadherin

AmotL2 has previously been shown to associate with VE- and E-Cadherin and is indispensable for actin filaments organization (Hildebrand et al., 2017; Hultin et al., 2014). Furthermore, it was evident from earlier studies that Par3 was also shown to associate with VE-Cadherin in endothelial cells (Iden et al., 2006). To evaluate whether Par3 mediates AmotL2 association with E-cadherin we used (human Keratinocytes) HaCat cells. Using co-immunoprecipitation we found that Par3 was associated with AmotL2, E-cadherin, catenins, MAGI-1 and β-actin. This connection could be disrupted when Par3 was silenced using siRNA. These

results suggest a role for Par3 in transporting AmotL2 to the AJ where it associates with the E-cadherin. This association then allows actin filament organization and connection to the neighboring cells within the tissue. Taken together, we conclude that the binding of AmotL2 to Par3 is required for the proper targeting of AmotL2 to cellular junctions.

Paper 2

The E-cadherin/AmotL2 complex organizes actin filaments required for epithelial hexagonal packing and blastocyst hatching

AmotL2 expression in epithelial tissues

In this paper we investigated the role of AmotL2 in epithelial cells. Our previous studies revealed that AmotL2 acts as a force transducer by associating with VE-cadherin at cell junctions. This association induces actin filament organization required for dorsal aorta development in zebrafish and mouse embryos (Hultin et al., 2014). To analyze whether AmotL2-mediated cytoskeletal organization is required for epithelial cell shape and packing, we firstly analyzed the general expression pattern of AmotL2 using a publicly available Genesapiens database. Analysis of AmotL2 mRNA levels in different organs indicated that AmotL2 is expressed in most organs with an exception for lymphoid, blood and bone marrow. Furthermore, analysis of around 755 human cell lines showed that Amotl2 is primarily expressed in epithelial cells.

To further analyze the role of AmotL2 in cell junctions, we depleted AmotL2 in epithelial cell lines including Mdck, Caco2 and HaCat using AmotL2 shRNA delivered by lentivirus. The efficiency of the shRNA AmotL2 was assessed by western blot. Immunofluorescence staining was used to analyze the junctional localization of the AJ protein E-cadherin and TJ protein ZO-1 upon depletion of AmotL2. Interestingly, we observed both E-cadherin and ZO-1 localized to the cell-cell junctions independently of AmotL2 levels. Several studies have previously shown that the Amot family proteins can bind to the polarity protein complexes Par3 and Crb3 (Ernkvist et al., 2009; Hultin et al., 2014; Wells et al.,

2006). We assessed whether silencing of AmotL2 negatively affected apical-basal polarity. The sorting of apical and basal markers did not seem to be affected as analyzed by immunofluorescent staining.

AmotL2 controls cell shape and epithelial packing in-vitro through actin filament organization

In multicellular tissues, epithelial cells are packed in a hexagonal and pentagonal fashion in order to efficiently transduce mechanical signals and to minimize energy expenditure on the epithelial surface. This patterning is unique to epithelial cells and is conserved among different species from plants to mammalian epithelium (Gibson et al., 2006; Gibson and Gibson, 2009; Hayashi and Carthew, 2004; Li et al., 2012). We measured the number of sides bordering neighboring cells in control and AmotL2 shRNA cells. We found that the majority of control cells are packed into hexagons and pentagons (with 5-6 sides in contact with other cells), whereas the AmotL2 shRNA cells bordered around three to four neighboring cells, and therefore exhibiting reduced contacts with neighboring cells. It has been previously reported that the junctional proteins together with actomyosin contraction control the length of cell junctions and cellular geometry (Gemp et al., 2011; Izaguirre et al., 2010; Lecuit and Lenne, 2007).

We further analyzed whether altered morphology of AmotL2 depleted cells could be due to the depletion of radial actin filaments. Using fluorescent microscopy, we showed that radial actin fibers, which run perpendicular to the cell cortex and are required for connecting neighboring cells, were lost in AmotL2 shRNA cells compared to controls. Interestingly, we did not note any changes in the cortical actin. We also treated MDCK cells with Blebbistatin (Myosin II inhibitor), which disrupts the actin filament assembly (Shutova et al., 2012). Treatment with blebbistatin for 2h induced changes in epithelial shape, which mimicked the phenotype of AmotL2 knock-down. Collectively, the above results suggest a role of AmotL2 in radial actin filament organization and control of cellular geometry and packing.

AmotL2 associates E-cadherin with actin cytoskeleton

We have previously reported that AmotL2 associates with VE-cadherin and the actin cytoskeleton together with other AJ proteins such as α and β catenins in endothelial cells (Hultin et al., 2014). In order to analyze this connection in epithelial cells we performed a series of co-immunoprecipitation experiments and found that AmotL2 associated with E-cadherin, α-catenin, β-catenin, MAGI-1 and actin. We were also able to map the interaction sites of AmotL2 to MAGI-1 to a previously described WW motif in the N-terminal domain (Bratt et al., 2005; Hultin et al., 2014; Patrie, 2005). This prompted us to investigate whether MAGI-1 serves as a direct connection between E-cadherin and AmotL2. Mutating the MAGI-1 binding domain of AmotL2 disrupted the interaction with β-actin but not E-cadherin, indicating an independent binding site for E-cadherin. To test this hypothesis we carried out co-immunoprecipitation using deletion mutants of AmotL2 spanning the N-terminal and C-terminal domains. This allowed us to map an 87 aa region in the N-terminal that could potentially facilitate the binding to E-cadherin. These findings suggest that MAGI-1, catenin complex, β-actin and E-cadherin interact with AmotL2 through separate domains.

AmotL2 is required for sensing the physical forces exerted upon epithelium

As described above AmotL2 induces the radial actin fibers, which also connect neighboring epithelial cells together and could potentially transmit mechanical signals throughout the epithelial layer. To test this hypothesis, stretch assays were performed on HaCat cells by growing them to confluency on an elastic silicone membrane coated with fibronectin. The silicone membrane is then stretched using a uniaxial stretch apparatus to apply mechanical stress to the HaCat monolayer. Following 2h of cyclic stretching, approximately 50% of control cells exhibit disrupted cell-cell junctions. The other 50% of control cells displayed rearranged actin filaments perpendicular to the direction in which the stretch was applied. In contrast, AmotL2 shRNA and blebbistatin treated epithelial layers remained intact, emphasizing that AmotL2-mediated actin fiber formation is essential for cells to respond to stretch stimuli applied to the

epithelial layer and as a result, the epithelium becomes more elastic and insensitive to the external forces.

AmotL2 controls cell shape and epithelial packing in zebrafish skin

Previous results indicated that knockdown of AmotL2 affected the cellular geometry and cell packing of Mdck cells. To further these observations *in vivo*, we analyzed the effects of AmotL2 depletion in zebrafish skin. In zebrafish, AmotL2 is expressed as two orthologues one localized in chromosome 6 (AmotL2a) and the other in chromosome 2 (amotL2b) (Hultin et al., 2014). We utilized AmotL2 MO to silence both AmotL2 orthologues in zebrafish skin. The knockdown efficiency of AmotL2 MO was evaluated by western blot analysis. By immunofluorescent staining we observed that AmotL2 was localized to the cell junctions and radial actin fibers in zebrafish skin. Silencing AmotL2 using MO resulted in the disruption of radial actin filaments. However, the cortical and junctional actin filaments were unaffected by AmotL2 knockdown. The localization of junctional proteins such as E-cadherin and ZO-1 were also analyzed by immunofluorescent staining. E-cadherin was found to be localized at cell junctions in both conditions, however, displayed diffuse localization in the AmotL2 knock-down zebrafish skin. Staining of ZO-1 indicated that cellular morphology of the skin was perturbed by AmotL2 knockdown, with increased cellular area and fewer sides bordering on neighboring cells. This phenotype could be rescued by co-injection of zebrafish with AmotL2 MO together with human p100AmotL2, which restored cellular geometry and packing in zebrafish skin to that of control zebrafish. These results, along with previous findings in Mdck cells suggest that cellular morphology and cellular packing of epithelial cells are disrupted in the absence of AmotL2. Collectively, these results emphasize the role of AmotL2 a crucial regulator of epithelial cell geometry and packing in zebrafish skin.

Amotl2 regulates blastocyst cell shape by radial actin fiber organization

The mammalian blastocyst consists of two cell types; the outer epithelial trophoectoderm (TE) and inner cell mass (ICM), where the TE is essential for

embryo implantation and later develops into the placenta. The ICM differentiates into the embryo proper and early endoderm (Hirate et al., 2013). Angiomotin family proteins (Amot and AmotL2) and the Hippo signaling pathway have previously been reported to be crucial in regulating the early cell lineage specification of mouse blastocysts (Hirate et al., 2013; Leung and Zernicka-Goetz, 2013). Furthermore, recently published data revealed that AmotL2 is highly expressed in the TE compared to the ICM in mouse and human blastocysts (Petropoulos et al., 2016; Posfai et al., 2017). The expression pattern of AmotL2 was analyzed using immunofluorescent staining in mouse and human blastocysts and indicated that AmotL2 is localized to the lateral cell-cell junctions in the TE. To further investigate the role of AmotL2 in the regulation of cell shape and size of the blastocyst, we silenced AmotL2 by injecting cre mRNA into fertilized zygotes of the floxed AmotL2 mouse strain using GFP mRNA injections as control (Hultin et al., 2014). AmotL2 gene inactivation was confirmed using immunofluorescent microscopy. As previous findings show that the Amot family proteins regulate the specification of cell lineage, we immunostained control and AmotL2 silenced mouse blastocysts with antibodies against CDX2 (a TE specific transcription factor). Interestingly, CDX2 staining was restricted to the outer TE of both blastocysts group. We further analyzed changes in cell number of the TE and ICM upon AmotL2 silencing and found that both cohorts contained equal number of cells, indicating that proliferation/cell division was unaffected by AmotL2 knockdown.

As already alluded to, we have shown that AmotL2 mediated radial actin fibers are implicated in regulating cellular geometry and packing in zebrafish skin and cell lines. To investigate the role of AmotL2 in blastocyst radial actin filament regulation, we performed phalloidin staining and confirmed the existence of both junctional actin and radial actin filaments in control blastocysts. As expected, AmotL2 silenced blastocysts exhibited a loss of radial actin filaments, whereas junctional actin remained intact. Furthermore, the loss of radial actin filaments resulted in an overall increase of cell area, volume and also affected the hexagonal packing of the TE cells in blastocyst. Similar results were observed upon treatment of mouse blastocysts with blebbistatin, which phenocopied AmotL2 silencing in blastocysts. These results confirmed a role for AmotL2 in the

regulation of radial actin filaments and concomitantly the cellular geometry and packing of cells in the TE of mouse blastocysts.

AmotL2 is essential for blastocysts hatching

The Zona pellucida, an acellular envelope, is a protective coat that surrounds mammalian embryos. Hatching out of the Zona pellucida is essential for uterine implantation and the continuous maturation of the embryo (Gupta et al., 2012; Leonavicius et al., 2018; Vasioukhin and Fuchs, 2001). The hatching of embryos from the zona pellucida is achieved through enzymatic digestion coupled with contraction and expansion of the blastocyst (Cohen and Feldberg, 1991; Hammadeh et al., 2011; Hiraoka et al., 2008). Moreover, previously published studies have emphasized the crucial role of actin fibers in the hatching of the blastocyst from the zona pellucida (Cheon et al., 1999; Suzuki and Niimura, 2010). We therefore investigated whether loss of radial actin filaments induced by silencing of AmotL2 would perturb the process of hatching. Interestingly, we observed that during *in vitro*-cultures 80% of AmotL2 silenced blastocysts were trapped in the zona pellucida, whereas 90% of control embryos were able to correctly progress to the hatching stage. These results suggest that AmotL2 dependent regulation of radial actin fibers is crucial in both early development and post developmental maintenance of cell morphology.

Paper 3

Uncoupling of the nuclear LINC-complex from E-cadherin/radial actin filaments trigger single cell migration and invasion

In Paper 2, we have shown that the longer isoform of AmotL2 (p100AmotL2) is a novel component of the E-cadherin/actin complex and that AmotL2 influences the regulation of actin filament, which is crucial in maintaining cell geometry and cell packing *in vitro* and *in vivo*. In this paper we have analyzed the specific effects of the shorter isoform of AmotL2 (p60AmotL2), focusing particularly on its role in regulating E-cadherin and cellular invasion.

AmotL2 expression in cancer patients

AmotL2 exists as two different isoforms, a longer (p100AmotL2) and a shorter (p60AmotL2) isoform encoded by separate promoters (Hultin et al., 2014; Mojallal et al., 2014). p100AmotL2 is constitutively expressed under normal physiological conditions, whereas p60AmotL2 expression is induced by severe hypoxia (*in vitro*) and ischemia (*in vivo*). Moreover, we have previously reported that AmotL2 expression was restricted to depolarized and invasive areas in human colon and breast cancer samples (Mojallal et al., 2014). In this paper, using immuno-histochemical staining, we have further evaluated AmotL2 expression in other cancers types including prostate, neuroendocrine and glioblastoma. Interestingly AmotL2 staining was limited to the invasive areas of the tumour, indicating a potential role for AmotL2 with respect to tumor invasion.

p60AmotL2 sequesters p100AmotL2 to intracellular vesicles and perturbs its normal functions

The members of Angiomotin protein family form oligomers by associating through their coiled-coil domains (Ernkvist et al., 2008; Moleirinho et al., 2014), which prompted us to speculate that p60AmotL2 may associate with p100AmotL2 and interfere with its molecular functions in a dominant-negative fashion. In order to test this hypothesis, we used MDCK cells transfected with a doxycycline (Dox) inducible p60AmotL2 construct and found that p60AmotL2

sequestered p100AmotL2 to intracellular vesicles when analyzed by immunofluorescent staining. Furthermore, co-immunoprecipitation studies using antibodies specific to the N-terminal domain of p100AmotL2 revealed an association between p100AmotL2 and p60AmotL2, E-cadherin, α & β-catenin, MAGI and actin. Furthermore, inducing p60AmotL2 expression disrupted the interactions of p100AmotL2. Immunofluorescent staining indicated that radial actin fibers were disrupted upon induction of p60AmotL2 expression resulting in altered cellular geometry and cell packing, similar to what we had observed previously in p100AmotL2 depleted cells (Hildebrand et al., 2017). From these results we concluded that p60AmotL2 acts as a dominant-negative inhibitor of p100AmotL2.

p60AmotL2 activates single cell invasion in 3D collagen matrix

The expression pattern of AmotL2 in patient cancer samples led us to speculate that p60AmotL2 expression may influence *in vitro* morphogenesis and promote invasion. To investigate this hypothesis, we grew single MDCK control and MDCK p60AmotL2 doxycycline inducible cells in a three dimensional (3D) collagen I matrix. MDCK cells grown in this setting proliferated and organized into cyst-like structures. Treatment with hepatocyte growth factor (HGF) stimulated these cyst-like structures to form tubules in control MDCK cells. Interestingly, doxycycline induced p60AmotL2 cysts exhibited a scattered and invasive migratory phenotype. The ECM provides necessary traction force for protruding cells during tube formation. We therefore analyzed interaction between the ECM and protruding tubes and scattering cells. This was performed by labeling of the collagen matrix with Oregon green. Results indicated that tubes of control cells interacted with the collagen matrix by extending the matrix-associated fibers. In contrast, p60AmotL2 expressing cysts and the associated scattering cells did not distort the surrounding collagen matrix, in the same manner observed with control cells. When observed in more detail by immunofluorescent microscopy, tubules of control cells were found to exhibit long actin filaments connecting the tip cell of the sprout to the supporting stalk cells. However, the disseminating cells of p60AmotL2 expressing cysts lacked these traversing actin fibers.

P60AmotL2 influences solidity of the nuclear membrane

We hypothesized that a direct link exists between the cell-cell junctions and nucleus, and therefore we went on to analyze the nuclear properties of the control and p60AmotL2 expressing cysts. As expected, the nuclei of control cysts elongated and migrated in the direction of protruding tubes, whereas the nuclei of p60AmotL2 expressing cells appeared disconnected from the actin cytoskeleton and displayed irregular contours compared to the controls. To further investigate the nuclear properties, we analyzed the nuclear lamins, and in particular Lamin A/C in the nucleus of protruding tubes and scattering cells. Lamin A/C has been shown to regulate the stiffness of the nuclear envelope (Lammerding et al., 2006). Immunofluorescent staining indicated that the intensity of Lamin A/C fluorescence was elevated in the protruding tip cell compared to the stalk cells, indicating that the nuclei of tip cells display greater malleability. In contrast, no significant difference in fluorescent intensity of Lamin A/C was observed in the p60AmotL2 cysts and subsequent invading cells. The above results strongly emphasize that the transmission of mechanical cues from cell junctions to nucleus is crucial for regulating nuclear integrity and that p60AmotL2 is a key regulator of such forces from the cell periphery to the nucleus.

p60AmotL2 abrogates the transmission of mechanical force from cell-cell junction to nuclear lamina

To measure the mechanical forces between the cell junctions and nuclear lamina, we utilized FRET based sensor probes for both E-cadherin and nesprin-2. FRET index exhibited by p60AmotL2 expressing cells indicated increased FRET index for both E-cadherin and nesprin-2 biosensors compared to control cells, suggesting that p60AmotL2 expressing cells exhibits decreased force in cell junctions and nuclear envelope. To corroborate our results obtained on nuclear rigidity, we used AFM to directly measure nuclear stiffness. Results indicated that the nuclear stiffness of p60AmotL2 expressing cells was greatly reduced in comparison to control cells. These data led us to hypothesize that lower mechanical force acting upon the nucleus of p60AmotL2 expressing cells would alter their physical properties and may promote cell migration through a dense

matrix. We therefore analyzed the migratory capacity of p60AmotL2 expressing cells using a transwell migration assay. We observed that approximately 80% of p60AmotL2 expressing cells were able to migrate across the 3-μm filter-pores, in comparison to 20% number of control cells, suggesting that p60AmotL2 cells were indeed more migratory. Collectively, our data identify a novel mechanism of E-cadherin regulation by the relative expression of the two AmotL2 isoforms. Furthermore, we show that the E-cadherin/p100AmotL2 interaction is required for the transmission of mechanical forces from the cell-cell junctions to nucleus via the actin cytoskeleton. Inactivation of this mechanotransductory complex alters cell-cell cohesions, nuclear integrity and promotes cellular invasion.

Paper 4

p60AmotL2 induces epithelial apical extrusion

Our previous work (Paper 2) has shown that a novel E-cadherin/p100AmotL2 mechanotransductory complex connects radial actin filaments to epithelial AJ´s. We have also shown that p60AmotL2 modulates E-cadherin function and also compromises nuclear integrity by disrupting radial actin filaments leading to cellular invasion (Paper 3). This final project aimed to elucidate the normal physiological function of p60AmotL2 in cellular morphogenesis.

p60AmotL2 expression in human cancers and extrusion prone mouse intestinal villi

Previous evidence presented here indicates that p60AmotL2 is localized to the invasive front of various tumors (Mojallal et al., 2014) and is able to modulate E-cadherin mechanotransduction (Paper 3). It is known that tumors hijack normal processes for their own benefit, this led to the question to what is the normal function of p60AmotL2. Immunohistochemical stainings provided some insight. Vesicular p60AmotL2 stainings localized intestinal areas characterized by high turnover of cells by the mechanism of apical extrusion. This is a process by which apoptotic or redundant cells are removed from an epithelial layer to maintain cellular homeostasis (Hogan et al., 2009; Kajita et al., 2010; Leung and Brugge, 2012). In order to test the above hypothesis, we analyzed the villus of mouse small intestine where the cellular turnover rates and apical extrusion are high

compared to other tissues. Immunohistochemical staining and western blot analysis revealed that p60AmotL2 expression is higher in the mouse intestinal villi (characterized by high levels of extrusion) as compared to the basal areas including the villus crypts characterized by high cell proliferation. This provided circumstantial evidence that p60AmotL2 may be involved in epithelial cell shedding.

p60AmotL2 expression induces apical extrusion *in-vitro*

To further investigate this phenomenon, we transfected MDCK cells stably expressing E-cadherin-tagged with red fluorescence protein (E-cad-RFP) with a p60AmotL2 construct labeled with green fluorescence protein (p60AmotL2-GFP). We also transfected a p60AmotL2 plasmid with a mutated PDZ binding domain and a myristylated-GFP plasmid as controls. Immunofluorescent time-lapse imaging over a period of 15 h showed that single p60AmotL2 expressing cells were readily extruded from the epithelial monolayer compared to the controls. Interestingly, p60AmotL2 positive cells in groups of >2 were not extruded from the epithelial monolayer, indicating that p60AmotL2 expression only promotes the apical extrusion of single cells. Previous studies have shown that contractile forces mediated by the actin cytoskeleton of neighboring cells are crucial in promoting the elimination of cells that are intended for extrusion (Kuipers et al., 2014; Rosenblatt et al., 2001). We have previously shown that p60AmotL2 acts in a dominant negative manner, by disconnecting radial actin filaments from E-cadherin/p100AmotL2 complex (Paper 3). Therefore, we next analyzed the effect of p60AmotL2-induced extrusion on the actin cytoskeleton. Actin filaments were visualized using phalloidin in p60AmotL2 and control transfected cells. Results indicated that radial actin filaments of control cells were interconnected across the monolayer. p60AmotL2 expressing cells exhibited disconnected radial actin filaments, which we anticipate, lead to the increased apical extrusion of p60AmotL2 expressing cells we previously observed. The above findings suggest a model whereby a decrease in contractile force in p60AmotL2 expressing cells could possibly trigger the apical extrusion of a p60AmotL2-expressing cell.

P60AmotL2 expression did not activate apoptotic program

It is possible that p60AmotL2 expression may induce apoptosis and so leads to apical extrusion. In order to address this question we performed colony formation assays, which showed that p60AmotL2 expressing cells formed viable colonies when cultured for a 7-day period. Additionally, immunostaining of caspase-3 indicated that p60AmotL2 positive extruding cells were negative for this apoptotic marker. These results suggest that p60AmotL2 transfection does not induce apoptosis *in vitro* and that p60AmotL2 induced apical extrusion is mediated by modulation to contractile actin.

Endogenous expression of p60AmotL2 correlates rosette formation

As previously described, p60AmotL2 is activated by stress signals such as hypoxia (*in vitro*) or ischemia (*in vivo*) (Mojallal et al., 2014). We have shown that in MDCK cells, p60AmotL2 sequesters p100AmotL2 into intracellular vesicles (Paper 3). In normal cell cultures, basal p60AmotL2 expression is very low and beyond detection levels. We therefore have utilized overexpression models in order to study the phenotype and mechanisms of p60AmotL2 expressing cells. The mechanism by which p60AmotL2 is activated on a single cell level remains to be studied in further detail. To explore the endogenous expression of p60AmotL2, we performed cell-overcrowding assay where untreated cells were plated in a very high density in order to activate a normal apical extrusion program. Analysis of immunostaining revealed that approximately 30% of extruding cells exhibited a vesicular pattern of AmotL2 staining, which is a characteristic feature of p60AmotL2 expression localization as previously described in paper 3. In conclusion, we have shown that p60AmotL2 expression induces the apical extrusion of single cells and speculate that this may provide an alternative mechanism by which tumor cells hijack normal cellular processes of cell extrusion to disseminate from normal epithelia and invade the surrounding tissues.

Concluding remarks and future perspectives

In this thesis, we have given mechanistic insights about how the neighboring cells are connected to each other via the cadherin transmembrane protein and the actin cytoskeletal networks. Furthermore, these cadherin-based connections span over a multicellular sheet and are crucial for sensing and resisting the incoming mechanical signals. Importantly, the transmission of mechanical forces from cell junction through cytoskeleton has been shown to regulate diverse developmental processes such as cell differentiation, morphogenesis, migration and gene expression. This thesis particularly focuses on AmotL2, which we elucidated as a linker protein connecting VE/E-cadherin from the AJ´s to the actin cytoskeletal networks in the cytoplasm. The above-elucidated connection mediated by AmotL2 enables the cells in a multicellular sheet to sense and adapt to the mechanical cues from the surrounding environment. In paper 1 & 2 of this thesis we have revealed how this mechanotransductory machinery might affect developmental processes ranging from dorsal aorta expansion in zebrafish to blastocyst hatching in mouse and human embryos.

In paper 3, we have showed that E-cadherin/p100AmotL2/actin complex further extends and associates with the nuclear lamina via LINC complex. Further, we also elucidate that expression of shorter isoform p60AmotL2 disrupts the above connection to the nucleus, resulting in deregulation of E-cadherin and compromised nuclear stiffness. Importantly, loss of E-cadherin and altered nuclear stiffness are the major regulator of cellular invasion during cancer metastasis. In paper 4, we have illustrated the normal physiological function for p60AmotL2. The loss of tensile force due to p60AmotL2 expression triggered neighboring cells to contract and apically extrude the affected cell. Since, p60AmotL2 is highly expressed in invasive cancer types suggesting that cancer cells may hijack this normal process of homeostasis to invade neighboring tissues, future studies on these lines will be vital in comprehending and curing metastatic cancers.

AmotL2 in treatment of atherosclerosis

Atherosclerosis was shown to develop due to the altered blood flow patterns inducing changes in the shear stress put forth on the endothelial cells in the artery walls(Heo et al., 2014). Importantly, shear stress also leads to the altered endothelial cell shape by modulating the actin cytoskeleton(Tzima et al., 2005). We showed previously that AmotL2 controls endothelial cell shape by organization of actin filaments (Hultin et al., 2014). Further, investigating the connection between shear stress and AmotL2 regulation would provide mechanistic insights to develop new therapeutics or preventive measures for atherosclerosis.

One of the common methods to treat severe atherosclerosis includes replacing the damaged vessel with saphenous vein graft (SVG). However, 50% of the SVG were shown to fail over the time span of 10 years indicating the need for improved grafting methods(Harskamp et al., 2013). A possible explanation for SVG failure could be the veins may not adapt to the high blood pressure in the arteries. In our lab using mice model, we are attempting to modulate the characteristics of sephanous vein prior to grafting by overexpressing AmotL2. We hypothesize that the high expression of AmotL2 would alter the sephanous vein to withstand high blood pressures as found in the arteries.

Identifying p60AmotL2 induced plasticity of 3D genome organization

In paper 3 we have highlighted a novel mechanotransductory pathway linking cell-cell junctions via p100AmotL2 and actin directly to the nuclear envelope, whereas p60AmotL2 disrupts the above connection. Furthermore, number of recent studies have highlighted that the mechanical signals transduced via cell junctions can also modulate chromatin organization and gene expression patterns within the cell (Miroshnikova et al., 2017; Uhler and Shivashankar, 2017). One possible mechanism by which mechanical forces could directly affect the chromatin organization can be *via*p100/p60AmotL2 mediated regulation of nuclear architecture which potentially modulate the 3D structure of chromatin, consequently leading to wide-scale gene expression changes. Since p60AmotL2 is linked to cancer, integrative study of mechanotransduction and genome architecture could prove to be invaluable to understand the basis of cellular

morphogenesis. More importantly this information could lead us to develop novel diagnostics and therapeutics for diseases like cancer.

To summarize, intercellular connections allowing mechanotransduction are crucial for assembly of individual cells into functional organs. Alterations occurring in the mechanosensory protein complexes can lead to loss of tissue architecture and also can promote aberrant gene transcription leading to various diseases. Therefore, understanding the mechanisms underlying the transmission of mechanical cues is of prime importance to avert and develop novel therapeutic tools for treating diseases caused by alterations in mechanotranscutory structures.

Acknowledgements

“Wherever we travel to, the wonderful people we meet become our family.”

(*Lailah Gifty Akita*)

I am very fortunate to get to know so many wonderful people during my PhD journey at Karolinska Institute. This thesis would not have been possible without the inspiration and support from you people. Thanks for all the good times!

Allt börjar med en bra lärare

I owe my deepest gratitude to my main supervisor, **Professor. Lars Holmgren** for his immense support during my PhD studies. I always admired your unparalleled energy and leadership skills. Thanks for your vision, trust, guidance and motivation. You made the most of my successes, rescued my failures and made them good. Thanks a ton.

Besides my main supervisor I would like to thank My co-supervisors, **Jonas Fuxe** and **Johan Hartman** for always being available and providing the needed support during my Ph.D studies.

I would also like to thank, my PhD examination board members, **Kaisa Lehti, Ingvar Ferby, Mimmi Shoshan,** my external examiner **Marius Sudol** and defense chairperson **Daniel Hägerstrand** for kindly accepting the request to be in the thesis committee**.**

Special thanks to my first supervisor, **Bhavesh (Holmgren group)** for accepting me for my master thesis and patiently teaching me about the projects. Thanks once again for introducing me to the research world. My mentor for Phd studies **Soniya Dhanjal,** for being positive and for your moral support.

Thanks to all members of Holmgren group (Present and past)
Yuan, you are more than a colleague and one of my best friends in Sweden. Thanks for being extremely supportive. I always admire your hard work and time management skills. I wish you a good luck for your upcoming paper and PhD defense. **Evelyn,** thanks for your support in proof reading my thesis. I admire your dedication for everything you do. Good luck for your future ventures. **Pedro,** you are like a brother to me. You have become one of my closest friends in Sweden and now you are my colleague. It feels great to work with you in the same lab, good luck for building your light saber and your PhD. **Vicky,** for the good team works in the extrusion paper. You are very talented and you will definitely go far. Good luck! **Aarren**, I am very fortunate to have you in this time of PhD defense. You have shaped up the language very well in my manuscripts and thesis. Thanks a ton and whenever I read my thesis, I will remember you. Good luck! **Yu Meng**, your hard work will surely pay you off. Good luck for rest of the years.

Sarah, Thanks for being extremely supportive during the time in CCK, and to all the fun times, beers, dinners and barbeques. I miss you so much. **Sebastian,** it was great working with you in the blastocyst paper. Thanks for the fun times and Friday beers. **Tomas**, big thanks for teaching me live imaging, you will be impressed with the developments in the clutch paper. I would also like to thank other former members of Holmgren group **Arindam, Tomas, Tanja, Zheng, Mahdi, Nathalie, Stephanie, Katha, Thananya, Sumako** and **Yu Cheng**.

Thanks to the friends in CCK, who made my years memorable

Karthik, you are one of my closest friends and well wisher**,** thanks for being with me in bad and good times, feels like we grew up together. All the very best machi, I miss you a lot. **Mathilde**, for all the advice about science and future careers, and also for the endless fun, beers, dinner, movie nights, early morning wine and barbecues, and of course for introducing me to Camembert. **Johanna**, you are such a caring person. Thanks for the fun times and good luck for your future endeavors. **Sophia**, I am very happy to have you again in the same office and good luck for your PhD studies. **Ran Ma,** thanks for the funny conversations and constantly motivating me to find a girl friend during the start of my PhD career. **Xinsong,** for being extremely helpful and for the fun times we had in and outside CCK. **Lisa,** thanks for your motivation during the start of my PhD and also for funny lunch conversations. I wish you a good luck. **Kristina Witt,** for being positive and supportive during my thesis writing.

I would also like to thank other fantastic friends in CCK, **Ishani** for being considerate and for your advices. **Alessandro,** thanks for the fun times and good luck with your preparation for thesis defense. **Angelos, Muyi, Satendra, Dalel, Patricia, Pinelopi, Vassilis, Dimitri, Nikos, Ali reza, Pedram, Linnea, Nathalie, Claire, Miguel, Edel** and **Masako** for the memorable times in CCK.

Thanks to the administration of Onc-Pat, especially **Erika, Hannah, Soren, Elisabeth, Eva lena,** for your immense support.

Thanks to collaborators
Daniel Conway, Vani Narayan, Malgorzetta for wonderful results in p60AmotL2 paper. **Per uhlen, Connala Edwards** for high-resolution imaging.

Friends outside work in Sweden

My close friends**, Roshan Vaid** thanks for being supportive right from the beginning of my masters degree till the end of my PhD. I have enjoyed your motivational talks, funny fights; endless caring for others and our friendship will last forever. All the very best for your PhD, it will be undoubtly fantastic. **Vivek,** thanks for your timely advices and I have enjoyed your company for almost 10 years now and will continue forever**. Mohan,** thanks for being an amazing friend and extremely helpful while in need. I will always cherish the trips we have made together**.** Good luck for your future. **Sateesh,** thanks for the weekend parties and

movie nights. Those were wonderful moments of my life. **Vairam**, thanks a lot for being my best buddy and for the wonderful trips and fruitful talks about life. Keep going with your search for knowledge. Good luck. **Johny,** you are a great and very close friend of mine, whom I can always count in, thanks for being around my friend! **Bala**, machi thanks for being with me for more than a decade, we shared all the fun times and tough times together. I am very fortunate to have you as my friend. I would also like to extend my thanks to **Avinash, Jai, Priya, Swetha, Ritika, Vinoth, Ganesh, michelle, Sharath, Moni, Shakthi, Sunitha, Kunal, Parvin, Christine, Bea** and **Ankit** during my journey in Sweden, you guys are simply fantastic!.

Dear Family,

Amma and **Appa,** thanks for your immense support and guidance throughout my life. It is impossible to explain your sacrifice in words, simply thanks for prioritizing my education over everything, your selfless love, support and trust made me to chase my passion. I hope I made you proud.

Anandhi and **Naveen,** thanks for all the help to Amma and Appa. Thanks for supporting them**.** Now you are blessed with **Narumugai** and the **little one,** I wish all the very best for you guys. I miss being with you. I would also like to thank **Chithapa, Thanaa, Hepsi, lincy, Sam, George** and **Hannah**. God bless you guys!

I would like to thank my dear wife **Roopa** for your patience and care, especially during the past three months. Hopefully, very soon you will move to Sweden and we can start our life happily. Love you Loads.

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